MATRIX METALLOPROTEINASES (MMPS) AND THEIR SPECIFIC TISSUE INHIBITORS (TIMPS) IN MATURE HUMAN ODONTOBLASTS AND PULP TISSUE

The regulation of expressions of fibrillar collagens, MMPs and TIMPs by growth factors, transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2)

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Institute of Dentistry,
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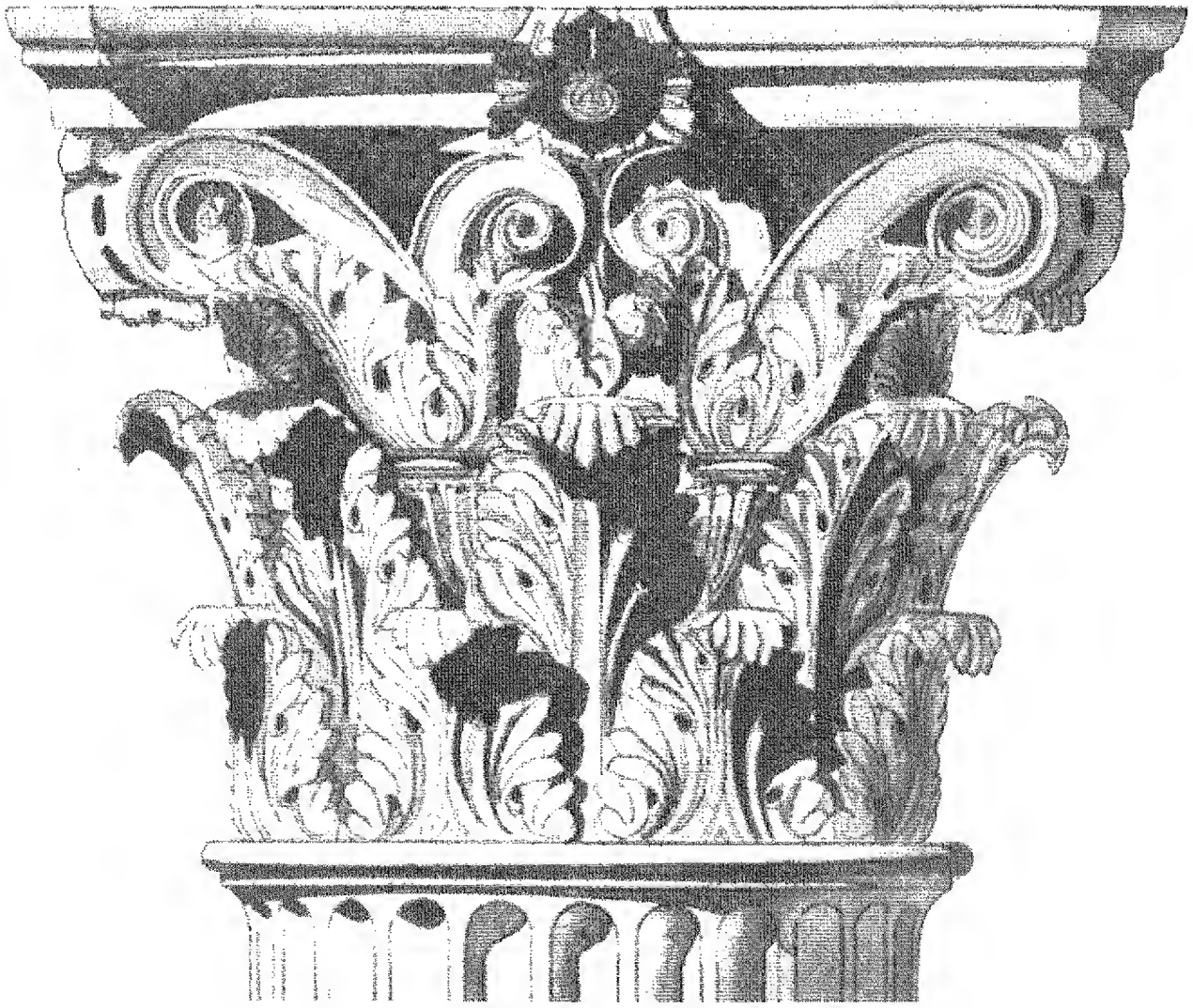

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Abstract

Dentin formation in physiological and pathological conditions has been widely studied, but the events and regulation are still not completely understood. Odontoblasts, terminally differentiated post-mitotic cells located in a single cell layer around pulp tissue, synthesize and mineralize dentin organic matrix. Growth factors, such as TGF-β1 and BMP-2, have been implicated in the regulation of the responses of odontoblasts and pulp tissue to external irritation. Matrix metalloproteinases (MMPs), a family of 28 endopeptidases collectively capable of degrading virtually all extracellular matrix components, and their specific tissue inhibitors (TIMPs) participate in the organo- and morphogenesis, physiological tissue turnover and pathological tissue destruction in many tissues, but very little is known about their presence, function, and regulation in the dentin-pulp complex cells and tissues. The aim of the work presented in this thesis was to analyze the expression and regulation of collagens, MMPs and TIMPs by TGF-β1 and BMP-2 in mature human odontoblasts and pulp tissue. Odontoblasts synthesize and secrete type I and type III collagens, with no clear effect of TGF-β1 on their expression levels. MMP-1, -2, -8, -9, -10, -11, -14, -15, -16, -19 and TIMP-1, -2, -3 and -4 were expressed by both odontoblasts and pulp tissue. MMP-3 and MMP-12 were not expressed in native odontoblasts or pulp tissue, and MMP-7, -24, and -25 were expressed only in odontoblasts. MMP-2, -10, -14, -20 and -23 were expressed more abundantly in odontoblasts, whereas pulp tissue expressed more MMP-13 and MMP-17. Growth factors differentially regulated the expression of different MMPs and TIMPs within and among the cells and tissues studied. In odontoblasts, MMP-1, -8 and -14 were down-regulated, but MMP-7, MMP-9, TIMP-1 and TIMP-3 up-regulated, by either TGF-β1 or BMP-2, alone or in combination. In pulp tissue, growth factors up-regulated the expression of MMP-1, -2, -10, -13, -17 and TIMP-3, but down-regulated TIMP-4. The widespread of expression of MMPs and TIMPs by mature human odontoblasts and pulp tissue suggests that they may participate in dentin matrix organization prior to mineralization, and that growth factors may further control dentin matrix modeling, not by regulating the synthesis of type I or III collagens as previously believed, but rather by differentially regulating each MMPs and TIMPs.

Keywords: dentin-pulp complex, human, MMP, odontoblasts, pulp tissue, TGF-β BMP-2, TIMP

To my mother Marja-Leena and my fiancé Timo

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New York, July 2003 Heidi Palosaari

Abbreviations

AP-1	activator protein-1
APMA	4-aminophenyl mercuric acetate
BM	basement membrane
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
C-	carboxy
cDNA	complementary DNA
C/EBP	cAMP element-binding protein
C/REB	cAMP response element-binding protein
cRNA	synthetic RNA
CT	cycle treshold
DAB	3,3-diaminbentzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF	epidermal growth factor
FGF	fibroblast growth factor
HGF	hepatocyte growth factor
IL	interleukin
kDa	kilodalton
MMP	matrix metalloproteinase
mRNA	messenger RNA
MT	membrane type
N-	amino
NCBI	national center for biotechnology information
NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEA3	polyomavirus enhancer A-binding protein-3 site
PDGF	platelet-derived growth factor
P1GF	placenta growth factor

PINP	type I procollagen
PIIINP	type III procollagen
PMA	phorphol 12-myristate 13-acetate
PMN	polymorphonuclear
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
TGF-β	transforming growth factor-β
TIE	TGF-β inhibitory element
TIMP	tissue inhibitor of metalloproteinase
TNF	tumour necrosis factor
TPA	12-O-tetradecanoyl phorphol 13-acetate
VEGF	vascular endothelial growth factor

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Palosaari H, Tasanen K, Risteli J, Larmas M, Salo T & Tjäderhane L (2001) Baseline expression and effect of TGF-β1 on type I and III collagen mRNA and protein synthesis in human odontoblasts and pulp cells in vitro. Calcif Tissue Int 68:122–129.

II Palosaari H, Wahlgren J, Larmas M, Rönkä H, Sorsa T, Salo T & Tjäderhane L (2000) The expression of MMP-8 in human odontoblasts and dental pulp cells is down-regulated by TGF-β1. J Dent Res 79:77–84.

III Palosaari H, Ding Y, Larmas M, Sorsa T, Bartlett JD, Salo T & Tjäderhane L (2002) Regulation and interactions of MT1-MMP and MMP-20 in human odontoblasts and pulp tissue *in vitro*. J Dent Res 81:354–359.

IV Palosaari H, Pennington CJ, Larmas M, Edwards DR, Tjäderhane & L Salo T (2003) Expression profile of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in mature human odontoblasts and pulp tissue. Eur J Oral Sci 111:1–11.

Contents

1 Introduction

In addition to minerals, which form about 70% (v/w) of the structure of dentin, the dentin-pulp complex of tooth consists of odontoblasts and pulp tissue. Odontoblasts are terminally differentiated, post-mitotic cells that synthesize and mineralize the organic matrix of dentin (Linde & Goldberg 1993). Dentin organic matrix consists mainly of type I collagen. Previous studies have indicated that matrix metalloproteinases (MMPs), a family of at least 28 endopeptidases collectively capable of degrading virtually all extracellular matrix components (Egeblad & Werb 2002), may exist in the dentin-pulp complex (Betti and Katchburian 1982, Dayan *et al.* 1983, Smith & Smith 1984, Fukae *et al.* 1991) and that some specific MMPs may participate to the regulation of dentin matrix mineralization (Hall *et al.* 1999). In addition, some of the MMPs have been detected in dentinal caries lesions (Dayan *et al.* 1983, Tjäderhane *et al.* 1998a). Dentin matrix also contains several growth factors, including transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-2 (BMP-2), which may regulate the secretory activity of mature human odontoblasts (Bessho *et al.* 1991, Cassidy *et al.* 1997) and play a major role in regulating the dentin-pulp complex responses to caries (reviewed in Tziafas *et al.* 2000).

Information on the expression and function of different MMPs, and their specific tissue inhibitors, TIMPs, in human dental tissues has been limited due to the lack of an *in vitro* model. Recently, an organ culture method of human odontoblasts and pulp tissue has been developed (Tjäderhane *et al.* 1998b). In this method, odontoblasts are cultured in the pulp chamber after removal of the pulp tissue, which itself can be further cultured separately. The method enables, for the first time, the study of the expression and regulation of proteins in mature human dentin-pulp complex. With the use of this organ culture method, the expression of different collagens, MMPs and TIMPs in mature human odontoblasts and pulp tissue were investigated. Their regulation by TGF-β1 and BMP-2 were also analyzed.

2 Review of the literature

2.1 Tooth structure

Macroscopically, a tooth is divided into a crown and a root (Fig. 1). The mature human tooth proper consists of three hard tissues; the crown is covered by enamel, which is hard and completely acellular and the most mineralized tissue found in the body. It consists of 95% inorganic hydroxyapatite crystallites ($Ca_{10}(PO_4)_6(OH)_2$) (all percentages as chemically by weight), the rest of the matrix being water (4%) and proteins (1%). The root is covered by cementum, which is 50% mineralized with hydroxyapatite crystals and has a collagen organic matrix. Below the enamel and cementum is a third hard tissue, dentin, which forms the bulk of the tooth. It is 70% mineralized with hydroxyapatite crystals, and organic material of the dentin is mainly fibrous collagen and comprises 20% of the matrix, while the remaining 10% is water. Mineralized dentin together with the pulp tissue form the dentin-pulp complex, which is responsible for the formation and maintainence of the tooth mass. (Ten Cate 1994a, Torneck 1994.)

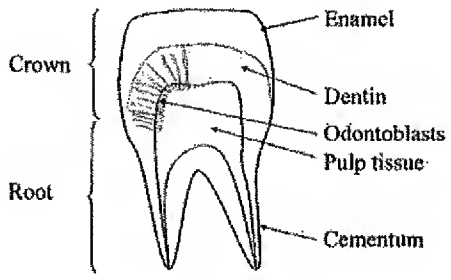

Fig. 1. A schematic picture of tooth structure.

2.2 Dentin-pulp complex

Dentin, the most voluminous mineralized connective tissue of the tooth, forms the hard tissue portion of the dentin-pulp complex, whereas the dental pulp is the living, soft connective tissue that retains the vitality of dentin (Linde & Goldberg 1993, Torneck 1994). Dentin contains multiple closely packed dentinal tubules in which the dentinal fluid and the cytoplasmic processes of the cells that have formed the dentin, the odontoblasts, are located (Torneck 1994). These most distinctive cells of the dentin-pulp complex lie along the predentin border forming the peripheral boundary of the dental pulp (see Fig. 1). Under the odontoblasts there is a cell-free zone (the zone of Weil), and beneath that a layer having high cell density (a cell-rich zone). The pulp core, in which the cell density is again reduced, consists of connective tissue, major blood vessels and nerves. The unity of dentin-pulp is responsible for dentin formation and protection of the tooth (Ten Cate 1994a).

2.2.1 Odontoblasts

Mature odontoblasts, which are located around the pulp chamber as a single cell layer, result from the differentiation of mesenchymal cells of the dental papilla during tooth development (Couve 1986, Ten Cate 1994c). Fully differentiated odontoblasts have withdrawn from the cell-cycle and are, thus, postmitotic cells in nature. Functionally active odontoblasts are polarized, having long cell bodies, which contain a well-developed granular endoplasmic reticulum, many mitochondria, a Golgi apparatus, a nucleus and several secretory vesicles (Linde & Goldberg 1993, Torneck 1994). At the distal end of the cell body, close to the predentin, odontoblasts are attached to each other by intercellular junctions, which enable communication between the cells (Ushiyama 1989, Bishop & Yoshida 1992). Peripheral to the intercellular junctions odontoblast cell processes arise and insert into dentinal tubules, crossing through the predentin zone to the mineralized dentin (Linde & Goldberg 1993). The cell processes lack major organelles involved in protein synthesis, but contain an abundance of longitudinally arranged microfilaments and microtubules. In addition, numerous vesicles reflecting both endo- and exocytosis traffic exist in the processes (Torneck 1994).

The main task of the odontoblasts is to synthesize and secrete collagens and several non-collagenous proteins of which the dentin organic matrix is formed. In addition, odontoblasts secrete signalling molecules, mainly of TGF-β superfamily (Cassidy *et al.* 1997), which are significant for cellular functionality (Bessho *et al.* 1991). Odontoblasts control dentin matrix mineralization at least by determining the nature of the extracellular matrix and by controlling the influx of mineral ions (Ten Cate 1985). After completion of primary dentin formation, odontoblasts transit into a resting state and their cell body structure transforms to a smaller and flattened type, with cellular structures changing their conformation or even disappearing (Couve 1986, Ten Cate 1994c). However, odontoblasts remain functional and still secrete and synthesize physiological secondary dentin, but at much slower rate. It is suggested that upon various stimuli, resting

odontoblasts are capable of up-regulating their secretory activity and responding by synthesizing tertiary dentin (Ten Cate 1994c).

2.2.2 Pulp tissue

Pulp organic matrix principally consists of type I and III collagens in a ratio of 55:45. In addition, ground materials such as glycosaminoglycans, glycoproteins and water comprise a supporting medium for cells and also for transportation of nutrients and other metabolites between the cells and vasculature (Torneck 1994).

Fibroblasts are the most abundant cell population of the pulp tissue, especially in the cell-rich zone (Torneck 1994). Their function is to form and maintain the pulp compartment. Other cell types of the pulp tissue are undifferentiated mesenchymal cells, macrophages and other immunocompetent cells. Also, vascular, nervous and lymphatic structures with their respective cell types are present. As a response to specific stimuli, such as caries or attrition, mesenchymal stem cells are believed to have the potential to differentiate into either odontoblast-like cells or fibroblasts (Torneck 1994). Odontoblast-like cells are responsible for the synthesis and secretion of reparative dentin under the circumstances where original odontoblasts are destroyed due to severe dental injury (D'Souza et al. 1995). In the pulpal steady-state condition macrophages are involved in the elimination of dead cells, whereas during inflammation macrophages remove bacteria and interact with other inflammatory cells to protect the pulp (Torneck 1994). Pulp tissue, however, is not responsible for the formation of physiological dentin of the tooth (Linde & Goldberg 1993).

2.3 Dentin formation and mineralization

Dentin structure and molecular chemistry may vary, even greatly, between different species. For example, the murine incisor tooth has ongoing dentinogenesis (Weinstock & Leblond 1973), and thus differs in the basis of dentin formation from that of the mature and fully developed human tooth. The formation of dentin is a highly regulated and controlled course of actions in which several constituents of both cellular and extracellular nature play a role. A complex compound of type I collagen fibres and a carbonate-rich apatite mineral phase ($Ca_{10}(PO_4)_6(OH)_2$) gives a quality of rigidity and strength to the dentin matrix (Ten Cate 1994b). In addition, intertubular dentin determines the elasticity of the dental matrix (Kinney *et al.* 1999).

2.3.1 Inter- and intratubular dentin

The predentin is an approximately 10–30 µm wide unmineralized zone between the mineralized dentin and odontoblasts, where dentin mineralization sharply occurs at a specific mineralization front (Jontell & Linde 1983, Linde & Goldberg 1993, Torneck 1994). At the onset of dentin formation, odontoblasts synthesize and secrete type I collagen and proteoglycans, and other significant constituents to the predentin layer. In predentin, collagen molecule fibers aggregate with their long axes in parallel into fibrils, which further arrange to bundles, possibly with the help of proteoglycans (Jones & Boyde 1984, Boskey 1989). A second, simultaneous process of formation of an inorganic phase at the mineralization front is pursued. Calcium ions (Ca^{2+}) are transported to the mineralization front by a transcellular route. This route includes a Ca^{2+}-activated ATPase, which in concert with Na^{+}/Ca^{2+} -exchangers, calcium channels and intracellular calcium binding proteins, maintain a delicate calcium ion homeostasis in odontoblasts (Granström & Linde 1981, Granström 1984, Carafoli 1987, Lundgren & Linde 1988, Magloire *et al.* 1988a). Concurrently, highly phosphorylated dentin phosphoprotein and dentin sialoprotein, phospholipids, and possibly another pool of proteoglycans, are added to the mineralization front, where they act as mineral nucleators and induce apatite formation. They bind to the collagen fiber surface and enhance the ability of the fibers to bind calcium ions and, in so doing, mineral deposition (Stetler-Stevenson & Veis 1986, Goldberg & Boskey 1996). Alkaline phosphatase, which non-specifically cleaves phosphate ions from compounds, is always associated with matrix mineralization, and its activity has been shown to increase at the mineralization front when crystal growth is occurring (Ten Cate 1994b). In addition, stromelysins of matrix metalloproteinases may further control proteoglycan turnover (Hall *et al.* 1999).

Primary dentin matrix is synthesized at a rapid rate during tooth development (Linde & Goldberg 1993, Torneck 1994). Thus, the tooth mass consists principally of primary dentin, which outlines the pulp chamber, and therefore it may be referred to as circumpulpal dentin. At the outermost layer of the primary dentin, just under the enamel, a narrow zone called mantle dentin exists. It is a product of the very first mineralization reaction by newly differentiated odontoblasts, and has slightly different composition than circumpulpal primary dentin. (Linde & Goldberg 1993, Torneck 1994.)

One characteristic feature of the physiological dentin matrix is its tubular pattern (Torneck 1994). Dentinal tubules may extend from the odontoblast layer to the dentin-enamel junction, and give high permeability to the dentin. In addition to an odontoblast process, the tubule contains dentinal fluid, a complex mixture of proteins such as albumin, transferrin, tenascin and proteoglycans (Linde & Goldberg 1993, Torneck 1994). Furthermore, dentinal fluid calcium and sodium concentrations have been shown to be higher in injured teeth compared to intact ones, indicating an active transportation system between the matrix and the tubulus (Larmas 2001). Loosely aggregated collagen fiber bundles have also been detected in the dentinal tubules (Dai *et al.* 1991). Whether the hypermineralized type of dentin detected inside the dentinal tubules (intratubular dentin) is the result of odontoblast activity or purely the result of physicochemical reactions within dentin minerals is still unclear (Torneck 1994). However, the intratubular dentin formation inside the tubules may ultimately obliterate the tubules, and reduce the

permeability of the dentin, which has, in turn, a positive impact on pulp vitality (Torneck 1994).

Following primary dentinogenesis, odontoblasts continue to deposit a physiological, secondary dentin around the pulp at slow rate leading eventually to the reduced size of the pulp chamber. Structurally, secondary dentin resembles primary dentin, also having a tubular pattern, which is, however, less regular than that of the primary dentin. (Linde & Goldberg 1993, Torneck 1994.)

2.3.2 Tertiary dentin

As a response to various external stimuli, such as dental caries, attrition and trauma, tertiary dentin is synthesized. If the injury is severe and causes odontoblast cell death, odontoblast-like cells synthesize specific reparative dentin just the beneath the site of injury to protect pulp tissue (Cox *et al.* 1992, Magloire *et al.* 1992, Lesot *et al.* 1993, Linde & Goldberg 1993, Torneck 1994, D'Souza *et al.* 1995). Unlike physiological dentin, the morphology of the reparative dentin varies greatly, and is often irregular, with cellular inclusions. Furthermore, the tubular pattern of the reparative dentin varies from a discontinuous to an atubular nature, and thus the reparative dentin matrix permeability is reduced and diffusion of noxious agents from the tubules is prevented (Tziafas *et al.* 2000). However, if injury to the tooth is mild and primary odontoblasts survive, they are stimulated to synthesize reactionary dentin, which resembles primary dentin matrix and has a tubular pattern (Tziafas *et al.* 2000).

2.4 Organic matrix of dentin

The dentin organic matrix primarily consists of fibrous collagens and other proteins such as proteoglycans, phosphoproteins and phospholipids, etc. The matrix provides a framework for mineralization. Collagens comprise 90% of the dentin matrix, and are principally type I (Gage *et al.* 1984, Lukinmaa & Waltimo 1992). Type I collagen is composed of two identical α1(I) chains and one α2(I) chain, and a glycine in every third amino acid position in an individual chain is needed for the formation of a triple helix structure (Beier & Engel 1966, Kielty *et al.* 1993). Proα2(I) mRNA has been shown to be expressed by mature human odontoblasts (Lukinmaa *et al.* 1992), whereas the expression of other collagen chain coding mRNA of proα1(I) has not been studied in fully developed human odontoblasts.

Type I collagen is synthesised as a larger procollagen, which contains extensions at both the N- and C-terminal ends, called the aminoterminal and carboxyterminal propeptides, which prevent premature collagen aggregation into fibrils. After procollagen secretion from cells, extracellular modification takes place, and propeptides are removed by specific proteinases and mature collagen molecules aggregate into a fibrous matrix (Kielty *et al.* 1993), which then serves as a support for mineral deposition.

Type I trimer, a more unusual type I collagen consisting of three α1(I) chains, is synthesised by odontoblasts actively forming mineralized dentin in rodent and bovine teeth. However, whether this occurs in the human tooth is unknown (Munksgaard 1979, Sodek & Mandell 1982).

Type III collagen, a homopolymer of three α1(III) chains, is a conspicuous constituent of soft connective tissues, such as pulp tissue, where it comprises approximately half of the collagen matrix (Shuttleworth *et al.* 1978, van Amerongen *et al.* 1983). In addition, there is strong evidence that calcified tissues are also able to express type III collagen, as Karjalainen and colleagues (1986) have shown that mature and intact human odontoblasts produce type III collagen after tooth development. Type III procollagen has been observed to be transiently located in human predentin during matrix formation, but not in mineralized dentin (Becker *et al.* 1986). The role of type III collagen in normal physiological dentin mineralization is unknown and awaits clarification. Type III collagen may be a more relevant constituent of the abnormal dental matrix, since it has been detected in dentinogenesis imperfecta patients (Waltimo *et al.* 1994). Type III collagen has also been detected in reparative dentin of carious human teeth (Karjalainen *et al.* 1986, Magloire *et al.* 1988b).

Additionally, other collagens may exist in the organic matrix of human dentin, since some expression of type V has been observed in the predentin of mature human teeth but not in dentin (Lukinmaa & Waltimo 1992). Instead, type VI was detected both in predentin and dentin of intact teeth (Becker *et al.* 1986), and it has also been found in the teeth of dentinogenesis imperfecta patients (Waltimo *et al.* 1994).

Proteins other than collagens comprise the remaining 10% of the dentin organic matrix. In addition, a minor part of the dentin matrix is composed of lipids, which possibly participate in mineral formation (Linde & Goldberg 1993). Of the noncollagenous proteins, dentin phosphoprotein (DPP; phosphophoryn) and dentin sialoprotein (DSP) represent the most abundant dentin-specific acid proteins in the dental matrix (Gu *et al.* 2000). A single gene encodes both, and after protein synthesis they are cleaved into the separate proteins of DPP and DSP. DPP is a highly phosphorylated protein, hydrophilic in character. It is capable of binding a large amount of calcium, facilitating the initial mineralization of the organic collagen frame. DPP is secreted by odontoblasts just ahead of the mineralization front (Butler *et al.* 1979, Takagi *et al.* 1986). The function of DSP is not yet known, but possibly it also has a role in the matrix mineralization reaction (Gu *et al.* 2000). Proteoglycans, such as decorin, bigclycan, fibromodulin and lumican, which carry glycosaminoglycan (GAG) carbohydrate side chains within their structures, comprise another sizeable portion of the noncollagenous proteins (reviewed in Embery *et al.* 2001). Since proteoglycans are also able to bind calcium (Embery *et al.* 1998), they may play a part in mineralization of the organic matrix of dentin, together with acid phosphoproteins.

2.5 Growth factors in dentin-pulp complex

Dentin matrix contains several growth factors and cytokines (see Table 1) with diverse biological effects on dentinogenic events in teeth (reviewed in Smith & Lesot 2001).

Since mature human dentin does not undergo remodeling like bone, it is likely that growth factors sequestered in the dentin matrix may mediate the cellular responses during tissue repair processes (Smith & Lesot 2001). The transformig growth factor-β (TGF-β) superfamily consists of numerous structurally related, secreted proteins including TGF-βs, bone morphogenetic proteins (BMPs), activins and inhibins (Wozney *et al.* 1988, reviewed in Massague 1990 and Risbridger *et al.* 2001). Generally, they play an essential role during development and maintain adult tissue homeostasis. In the dentin-pulp complex, TGF-βs or BMPs are believed to regulate both tooth development and the response to external irritation (Heikinheimo 1994, Nakashima 1994, Tziafas *et al.* 2000).

Table 1. Growth factors detected in human dentin-pulp complex.

Growth factor	Dentin matrix	Odontoblasts	Pulp cells or tissue
TGF-β1 [A,B]	+[A]	+[B]	
BMP activity [C]	+		
BMP-2 [D,E,F]		+[D]	+[E,F]
BMP-4 [E,F]			+
BMP-6 [E]			+
OP-1 [F]			+
EGF [G]	+		
FGF-2 [G]	+		
HGF [H]			+
IGF-I [I]	+		
IGF-II [I,J]	+[I]		+[J]
IL-1 [K]			+
IL-2 [L]			+
IL-6 [M]			+
IL-8 [N,O]		+[N]	+[O]
NGF [P]			+
PDGF-AB [G]	+		
PlGF [G]	+		
VEGF [G,Q]	+[G]		+[Q]

[A] Cassidy *et al.* 1997, Zhao *et al.* 2000
[B] Inage & Toda 1996
[C] Bessho *et al.* 1991
[D] Heikinheimo 1994
[E] Takeda *et al.* 1994
[F] Gu *et al.* 1996
[G] Roberts-Clark & Smith 2000
[H] Ohnishi *et al.* 2000
[I] Finkelman *et al.* 1990
[J] Shi *et al.* 2001
[K] D'Souza *et al.* 1989
[L] Rauschenberger *et al.* 1997
[M] Matsushima *et al.* 1998
[N] Levin *et al.* 1999
[O] Huang *et al.* 1999
[P] Woodnutt *et al.* 2000
[Q] Matsushita *et al.* 1999

2.5.1 Transforming growth factor-β1, TGF-β1

Of the five characterised TGF-β forms, TGF-β1, -β2 and -β3 have been identified in mammals, and so far only TGF-β1 is detected in human dentin (Cassidy *et al.* 1997, Zhao *et al.* 2000). In addition, TGF-β1 released from dentin matrix has target receptors both in odontoblasts and pulp tissue, indicating that signalling pathways in the cells respond to the TGF-β (Sloan *et al.* 1999). Specifically, TGF-β1 has been shown to associate with

reactions in response to the dental injury, by regulating tertiary dentin formation (Tziafas *et al.* 2000). Depending on the degree of injury, TGF-β1 may either stimulate pulpal cell proliferation and differentiation (Tziafas *et al.* 1995, Melin *et al.* 2000), or TGF-β may have effect on the odontoblasts and pulpal cell secretory activity (Smith *et al.* 1995, Melin *et al.* 2000). Depending on the cell responding to TGF-β1, reparative or reactive dentin is formed to protect pulp vitality against external irritants (Tziafas *et al.* 2000).

There are controversial views of the effect of TGF-β on type I collagen synthesis in calcified tissue repair. Although TGF-β may induce type I collagen synthesis in several tissues, in osteoblasts collagen synthesis is affected by the state of cell differentiation, and in mature osteoblasts TGF-β1 has no effect on collagen synthesis (Shibata *et al.* 1993). In addition, animal studies with osteoblast-like cells indicate that TGF-β1 has no major effect on type I collagen synthesis (Ibbotson *et al.* 1989). However, TGF-β may stimulate type I collagen in young cells, which have recently differentiated (Shibata *et al.* 1993, Melin *et al.* 2000).

2.5.2 Bone morphogenetic protein-2, BMP-2

Bone morphogenetic proteins (BMPs) are characterized as proteins being able to induce bone and cartilage formation (Wozney *et al.* 1992, Centrella *et al.* 1994), and possibly also formation of dentin, since unidentified BMP has been purified from human dentin (Bessho *et al.* 1991). At least 15 BMPs exist (Wozney *et al.* 1988, Celeste *et al.* 1990, 1995, Özkaynak *et al.* 1990, Storm *et al.* 1994, Song *et al.* 1995, Dube *et al.* 1996) of which BMP-2 and BMP-4 form a subgroup. BMP-2 represents a classical prototype of the BMPs, and it is expressed by both functional odontoblasts and human adult pulp tissue (Heikinheimo 1994, Gu *et al.* 1996, Calland *et al.* 1997). BMPs may have a special role in repair processes in mature teeth, since it has been shown that BMP-2 may induce reparative dentin formation (Nakashima 1994).

2.6 Dentin demineralization and destruction

The most common tooth structure-destructing condition, dental caries, is perhaps the most widely spread infectious disease in the world. It is a bacterial disease in which oral microbes produce acids such as lactate, acetate and propionate, which dissolve inorganic minerals, hydroxyapatite crystallites, first from the enamel, and then from dentin (Hojo *et al.* 1991, van Houte 1994). As a consequence of an acidic environment, the collagenous matrix of dentin is demineralized, leading to a caries lesion. However, bacterial acids are not able to hydrolyze fibrous collagens, and there is no evidence that bacterial enzymes are associated with caries development by degrading the dental organic matrix. On the contrary, *in vitro* experiments indicate that caries-related bacteria can readily demineralise the matrix, but fail to degrade the dentin organic matrix (Katz *et al.* 1987, van Strijp *et al.* 1997). Therefore, it has been suggested that host enzymes, matrix metalloproteinases,

degrade the dentin matrix during or after demineralization by bacterial acids (Tjäderhane *et al.* 1998a, Sulkala *et al.* 2001). Such host proteases, either from odontoblasts or pulp tissue may be responsible for collagen matrix destruction, and in combination with additional hydrolytic enzymes such as lactate dehydrogenase (LDH) and glycoproteases, may catalyze dentin connective tissue degradation in caries progression. In physiological conditions, proteolytic enzymes may in turn model calcified matrix. (Larmas 2001.)

2.7 Metalloproteinase superfamily

Proteolytic enzymes are either exopeptidases, cleaving a substrate molecule's terminal peptide bond, or endopeptidases, cleaving an internal peptide bond of the substrate (Stöcker *et al.* 1995). Endopeptidases are divided into serine, cysteine, aspartic and metalloproteinases based on their catalytic properties and inhibitor sensitivities. The metzincin superfamily, which belongs to the metalloproteinases, encode a highly conserved zinc-binding motif containing three histidine residues which bind zinc, and a conserved methionine-turn in the active-site helix. The metzincin superfamily includes serralysins, astacins, adamalysins and matrix metalloproteinases (MMPs) (Stöcker *et al.* 1995.)

2.8 Matrix metalloproteinases (MMPs)

MMPs comprise a family of at least 28 secreted or transmembrane enzymes collectively capable of processing and degrading various ECM proteins. Of these, at least 22 MMPs have so far been found to be expressed in human tissues. MMPs share high protein sequence homology and have defined domain structures and thus, according to their structural properties, MMPs are classified either as secreted MMPs or membrane anchored MMPs, which are further divided into eight discrete subgroups. Secreted MMPs include minimal-domain MMPs, simple hemopexin domain-containing MMPs, gelatin-binding MMPs, furin-activated secreted MMPs and vitronectin-like insert MMPs, while membrane bound MMPs include type I transmembrane MMPs, glycosyl-phosphatidyl inositol (GPI)-linked MMPs and type II transmembrane MMPs (see Fig. 2). (Reviewed in Egeblad & Werb 2002.)

Crystal structures of MMPs further uncovered the exact domain organization, polypeptide fold and main specificity determinants (reviewed in Bode *et al.* 1999). To date, crystal structures of the catalytic domains of human MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-11, MMP-12, MMP-13 and MMP-14, in addition to porcine full length MMP-1 and human proMMP-2 have been resolved (Van Doren *et al.* 1993, Reinemer *et al.* 1994, Bode *et al.* 1994, Lovejoy *et al.* 1994, Li *et al.* 1995, Fernandez-Catalan *et al.* 1998, Kiyama *et al.* 1999, Morgunova *et al.* 1999, Moy *et al.* 2000, Gall *et al.* 2001, Lang *et al.* 2001).

All MMPs are synthesized with a predomain containing a leader sequence, which targets the protein for secretion (reviewed in Sternlicht & Werb 2001). They are secreted as latent proforms, with a few exceptions of furin-processed proteinases, such as MMP-11 or MMP-28. The prodomain of MMPs has an egglike shape, and contains a well conserved cysteine switch motif of PRCXXPD for maintaining the proMMP latent (Springman *et al.* 1990, Van Wart & Birkedal-Hansen 1990). Generally, the structures of all MMP catalytic domains are quite similar (Bode *et al.* 1999). The shape of the catalytic domain is spherical with a flat active site cleft, which extends horizontally across the domain to bind peptide substrates or inhibitors. The catalytic domain has the zinc-binding motif, **H**EF/LG**H**S/AL**G**LX**H**S, which coordinates a zinc atom at the active site, and under the zinc, a ALMYP methionine-turn (Stöcker *et al.* 1995). The latency of the zymogen is maintained through cysteine-switch motif, in which the cysteine residue acts as a fourth zinc-binding ligand to maintain the enzyme inactive. In addition to the catalytic zinc, the catalytic domain also contains a structural zinc and two to three calcium ions. A sub-site- or S1'-pocket- or channel-like structure is a binding site for a substrate or inhibitor molecule within the active site, and differs considerably in size and shape among the various MMPs. P1' indicates the residue of a bound substrate molecule. The P1'-S1' interaction mainly determines the affinity of inhibitors, and the cleavage positions of peptide substrates. C-terminal hemopexin or vitronectin-like domains affect substrate or inhibitor binding, membrane activation and some proteolytic activities. The hemopexin domain, very similar in structure among the MMPs, is an ellipsoidal disc, and is connected to the catalytic domain by a hinge region. The hinge region is flexible and rich in proline residues. It may also influence substrate specificity. (Reviewed in Bode *et al.* 1999, Sternlicht & Werb 2001.)

Structural specificities among the different MMP subgroups can be recognized. Of the simple hemopexin domain-containing MMPs, which have the general structure of a preprodomain and a catalytic domain, which is connected through hinge to the hemopexin like-domain, at least MMP-1, MMP-8 and MMP-13 contain three conserved amino acids, Tyr-214, Asp-235, Gly-237, which are not present in other MMPs (Freije *et al.* 1994). Gelatin-binding MMPs have a unique 19 kDa fibronectin-like insert in the catalytic domain. This central domain is organized into three internal repeats, which are homologous to the type II motif of the collagen binding domain of fibronectin, and are needed for binding and cleaving collagen and elastin (Collier *et al.* 1988, 1992, Murphy *et al.* 1994, Shipley *et al.* 1996). MMP-9 has an additional, unique 54 amino acid long proline rich domain, not existing in other MMPs, which is homologous to the α2 chain of type V collagen (Wilhelm *et al.* 1989). Furin-activated secreted MMPs (MMP-11 and MMP-28) have a recognition motif for furin-like serine proteinases within their catalytic domain for intracellular activation. This motif is also found in the vitronectin-like insert MMPs (MMP-21), and the MT-MMPs (Egeblad & Werb 2002).

Type I transmembrane MMPs include MMP-14, MMP-15, MMP-16 and MMP-24, and have a carboxy-terminal, single-span transmembrane domain and a short cytoplasmic C-terminal tail (Kojima *et al.* 2000). MMP-17 and MMP-25 are anchored to the membrane by a C-terminal hydrophobic region with a glycosyl-phosphatidyl inositol (GPI) domain, and thus are classified as glycosyl-phosphatidyl inositol (GPI)-linked MMPs (Itoh *et al.* 1999, Kojima *et al.* 2000).

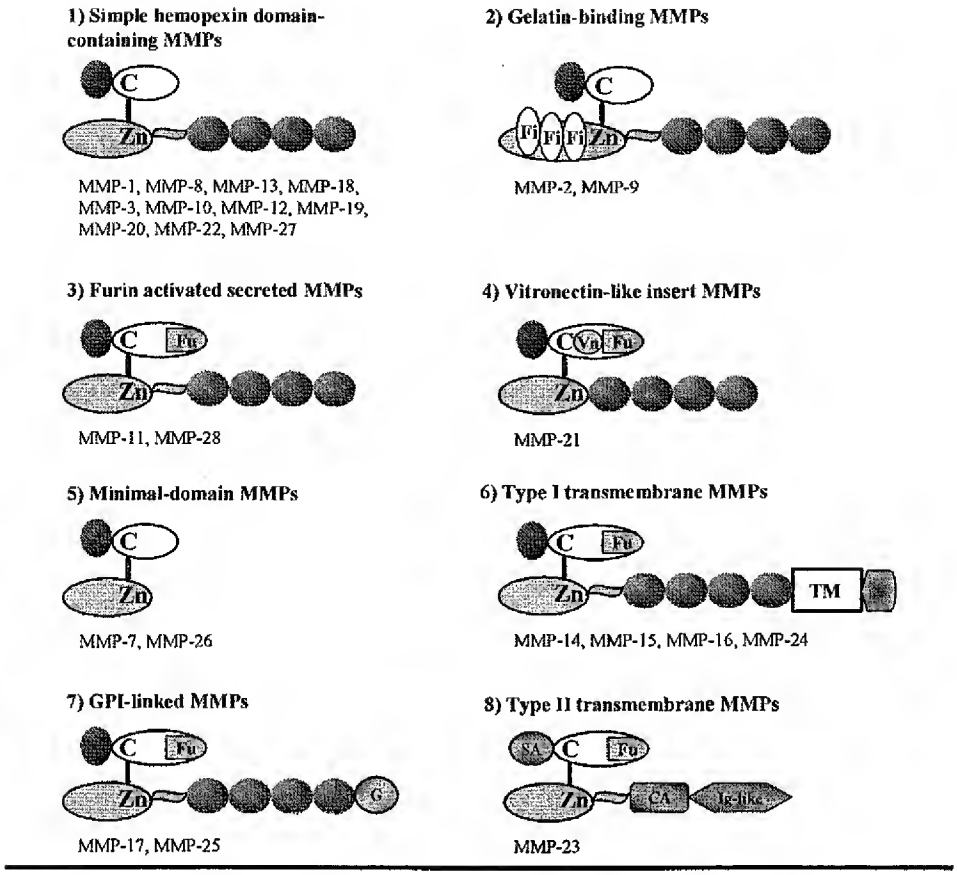

Fig. 2. Domain structure of MMPs and their classification. MMPs are illustrated as latent zymogens with a catalytic site zinc (Zn) binding to cysteine (C) of cysteine-switch of the prodomain. (Modified from Egeblad & Werb 2002)

Both classes of carboxy-terminal domains localize all of these MT-MMPs in a very specific way to the cell surface, therefore playing an important role in diverse proteolytic

events. MT-MMPs also have an eight amino acid insertion in the catalytic domain, which distinguish them from other MMPs (English *et al.* 2001). Analysis of this insertion in MMP-14 (163-PYAYIREG-170) indicates its importance for proMMP-2 activation (English *et al.* 2001).

Minimal-domain MMPs, MMP-7 and MMP-26, lack a hemopexin domain (Muller *et al.* 1988, de Coignac *et al.* 2000), whereas MMP-23 has cysteine array and immunoglobulin (Ig)-like domains instead of the conserved hemopexin-like domain (Pei *et al.* 2000). MMP-23 is also classified as type II transmembrane MMP, since it has an amino-terminal signal anchor (CA) targeting it to the cell membrane (Pei *et al.* 2000).

2.8.1 Simple hemopexin domain-containing MMPs

2.8.1.1 Collagenases

The story of MMPs begun when Gross and Lapiére (1962) detected collagenolytic activity in tissues of tadpoles, and called the respective enzyme collagenase. In oral tissues, this first characterized collagenase, MMP-1 (collagenase-1, interstitial collagenase, fibroblast collagenase), is detected in gingival fibroblasts capable of disrupting ECM collagen (Wilhelm *et al.* 1984). The MMP-1 gene has three dimorphic sites in the 5' upstream region, which may affect the regulation of gene expression (Thiry-Blaise *et al.* 1995). The promoter region of the human MMP-1 gene contains AP-1 and ETS elements, which are able to mediate at least PMA induction (White *et al.* 1997). Analysis of the rabbit MMP-1 TGF-β inhibitory element (TIE) in the promoter region, which is also conserved in the human MMP-1 gene, reveals that the TIE represses constitutive MMP-1 gene transcription (White *et al.* 2000). The MMP-1 gene is transcribed into 2.5 kb mRNA (Wilhelm *et al.* 1986).

Latent MMP-1 has two major species of molecular mass, 57 kDa and 52 kDa, in a ratio of 1:4 in tissues (Wilhelm *et al.* 1984). The minor 57 kDa proMMP-1 is a result of N-glycosylations of the major 52 kDa form (Wilhelm *et al.* 1986). Active MMP-1 exists in forms of 48 kDa and 42 kDa, of which the 42 kDa MMP-1 represents the stable, active enzyme (Wilhelm *et al.* 1984, Grant *et al.* 1987). MMP-1 collagenolytic activity is essentially mediated through the (183)RWTNNFREY(191) motif of the catalytic domain in concert with the C-terminal hemopexin domain (Chung *et al.* 2000). Active MMP-1 hydrolyzes type I collagen into N-terminal ¾, and the C-terminal ¼ fragments. However, it hydrolyzes type III collagen 10-fold faster than type I collagen (Wilhelm *et al.* 1984). MMP-1 also cleaves α-chains of native type II collagen (Fields *et al.* 1987), type VII collagen (Seltzer *et al.* 1989), type X collagen (Schmid *et al.* 1986), type VIII (Gadher *et al.* 1989), α2-macroglubulin (Sottrup-Jensen & Birkedal-Hansen 1989), gelatin and casein (Fields *et al.* 1990), α1-proteinase inhibitor and α1-antichymotrypsin (Desrochers *et al.* 1991), tenascin (Imai *et al.* 1994) and IL-1β (Ito *et al.* 1996).

MMP-8 (collagenase-2) represents the second collagenase, and was originally thought to be synthesised and stored exclusively in intracellular granules of human polymorphonuclear neutrophils (PMNs) in bone marrow (Bainton *et al.* 1971, Mainardi *et*

al. 1991). MMP-8 has been purified from these granules, from which the PMNs secrete the enzyme, and it has been described as neutrophil type or polymorphonuclear type (PMN) MMP-8 (Hasty *et al.* 1986). Mesenchymal type MMP-8, differing from neutrophil MMP-8 in protein size, is expressed by human chondrocytes (Cole *et al.* 1996), rheumatoid synovial fibroblasts and endothelial cells (Hanemaaijer *et al.* 1997). It is also produced in keratinocytes, including oral squamous cell carcinoma cells (Bachmeier *et al.* 2000, Moilanen *et al.* 2002) and plasma cells (Wahlgren *et al.* 2001). Furthermore, MMP-8 has been found in human gingiva (Tonetti *et al.* 1993), saliva (Ingman *et al.* 1994), dental plaque (Sorsa *et al.* 1995) and demineralised dentinal caries lesions (Tjäderhane *et al.* 1998a).

A 3.3 kb MMP-8 mRNA species codes for both neutrophil and mesenchymal type protein forms (Hasty *et al.* 1990, Hanemaaijer *et al.* 1997). It has been reported that a splice variant of the MMP-8 transcript exists. A cDNA with 91 bp insertion in its sequence still codes for the active MMP-8 protein. However, it is not secreted from cells (Hu *et al.* 1999). MMP-8 shares 57% identity to the MMP-1 protein sequence (Hasty *et al.* 1990). It contains eight possible glycosylation sites (Hasty *et al.* 1990), and hence several molecular weight forms of the MMP-8 protein have been published. The PMN type proMMP-8 has been reported to be 75–85 kDa in size. Active MMP-8 may be of 45 kDa, 57 or 64 kDa, respectively, in addition to smaller 22–28 kDa species (Hasty *et al.* 1986, Tschesche *et al.* 1992). The size of the chondrocyte proMMP-8 is 55 kDa and active forms are 46 and 42 kDa (Cole *et al.* 1996). MMP-8, secreted from the rheumatoid synovial fibroblasts and endothelial cells, is 50 kDa in size (Hanemaaijer *et al.* 1997).

MMP-8 degrades type I collagen into characteristic ¾ and ¼ fragments (Hasty *et al.* 1987, 1990). It also cleaves types II and III collagen molecules, yet with slower catalytic rates than type I collagen (Hasty *et al.* 1987). MMP-8 may also cleave ECM molecules other than triple helix collagens. It is able to cleave aggrecan (Arner *et al.* 1997), plasma serine-proteinase inhibitor, α_1-antitrypsin (Michaelis *et al.* 1990) and fibrinogen (Hiller *et al.* 2000).

MMP-13 (collagenase-3) was originally cloned from human breast tumour (Freije *et al.* 1994). The expression of MMP-13 is not restricted to breast tumours, but is also expressed by human chondrocytes (Blavier & Delaisse 1995, Mitchell *et al.* 1996, Reboul *et al.* 1996, Borden *et al.* 1996), synovial membrane (Wernicke *et al.* 1996), synovial stroma (Lindy *et al.* 1997), synovial fibroblasts (Westhoff *et al.* 1999), gingival fibroblasts (Ravanti *et al.* 1999) and plasma cells (Wahlgren *et al.* 2001). It is also expressed by hypertrophic chondrocytes, osteoblasts, periosteal cells and fibroblasts during human fetal bone development (Johansson *et al.* 1997, Stahle-Backdahl *et al.* 1997), and postnatally in bone remodeling (Stahle-Backdahl *et al.* 1997).

MMP-13 is transcribed into three different mRNA species with respective sizes of 2, 2.5 and 3 kb (Freije *et al.* 1994, Reboul *et al.* 1996). The human MMP-13 gene promoter contains recognition sites for TATA and CCAAT DNA-binding proteins, an AP-1 motif, PEA-3 consensus sequence, an osteoblast specific element (OSE-2), a TGF-β inhibitory element (TIE) and three motifs of hormone response elements (Pendas *et al.* 1997a, Tardif *et al.* 1997). Co-operation of SMAD proteins with the functional AP-1 site, in concert with PEA-3, is essential for both basal and inducible gene transcription, *e.g.* by TGF-β in chondrocytes (Tardif *et al.* 2001). MMP-13 has an AG-rich element (AGRE) in the proximal promoter region, which represses basal transcription of the gene (Benderdour *et*

al. 2002). A cDNA of MMP-13 codes for a 471 amino acid polypeptide. It has three potential N-glycosylation sites within an active protein molecule (Freije *et al.* 1994). The molecular weight of the proMMP-13 is 60–65 kDa. Active MMP-13 is 50–55 kDa in size (Freije *et al.* 1994, Knäuper *et al.* 1996a), but is further cleaved into a final active form of 48 kDa (Knäuper *et al.* 1996a).

Active human MMP-13 preferentially hydrolyses fibrillar type II collagen (Knäuper *et al.* 1996a, Mitchell *et al.* 1996, Reboul *et al.* 1996), and type II procollagen, both at the telopeptide and N-proteinase sites (Fukui *et al.* 2002). It also cleaves gelatin (Knäuper *et al.* 1996a) and cleaves type I and type III collagens into characteristic ¾ N-terminal and ¼ C-terminal fragments (Freije *et al.* 1994, Knäuper *et al.* 1996a). MMP-13 cleaves type II collagen approximately 10 times faster (Mitchell *et al.* 1996) and gelatin about 40 times more efficiently than MMP-1 or MMP-8, while type I collagen is cleaved with comparable efficiency to the MMP-1 and MMP-8 (Knäuper *et al.* 1996a). In addition, MMP-13 degrades types IV, IX, X and XIV collagens, tenascin, fibronectin, fibronectin fragments (Knäuper *et al.* 1997), cartilage proteoglycan, aggrecan (Fosang *et al.* 1996), plasma proteins, fibrinogen and Factor XII (Hiller *et al.* 2000).

MMP-18 (collagenase-4) was identified in *Xenopus laevis* (Stolow *et al.* 1996), but a human counterpart for this amphibian collagenase has not yet been identified. *Xenopus* collagenase-4 is 54% identical with human MMP-1. It contains the highly conserved cysteine-switch motif, except that proline is replaced with tyrosine. It degrades gelatine, and cleaves type I collagen into characteristic ¾ and ¼ fragments. (Stolow *et al.* 1996.)

2.8.1.2 Stromelysins

MMP-3 (stromelysin-1) was purified and characterized from human rheumatoid synovial fibroblasts (Okada *et al.* 1986) and skin fibroblasts (Wilhelm *et al.* 1987). The promoter of MMP-3 contains three elements important for mitogenic induction: a SPRE site (stromelysin-1 PDGF-responsive element) (Sanz *et al.* 1994), a PEA3 site (polyomavirus enhancer A-binding protein-3 site) (Wasylyk *et al.* 1991, Buttice & Kurkinen 1993) and an AP-1 site (activator protein-1 binding site) (Kerr *et al.* 1988). SPRE is the binding site for a transcription factor, SPBP (SPRE-binding protein), which has the ability to enhance transcription of other transcription factors such as c-Jun, Ets1, Sp1 and Pax6 (Rekdal *et al.* 2000). The PEA-3 site mediates TPA induction of human MMP-3 gene transcription (Buttice & Kurkinen 1993). The AP-1 site in the promoter of MMP-3 mediates basal gene expression, but is not necessary for the PMA-response of human MMP-3 (Buttice *et al.* 1991). Animal studies have revealed that the TGF-β1 inhibitory element (TIE) in the promoter region mediates the TGF-β1 inhibitory effect on stromelysin gene expression (Kerr *et al.* 1990). However, the age of the cell population affects whether MMP-3 is induced or repressed by TGF-β1. In young fibroblasts, TGF-β1 represses the gene induction, whereas in older cells, repression is not apparent (Edwards *et al.* 1996).

MMP-3 is 54% identical to human MMP-1 and 87% homologous to its rat counterpart (Saus *et al.* 1988). ProMMP-3 is secreted as a 57 kDa form, which can be glycosylated into a 60 kDa protein (Wilhelm *et al.* 1987). Latent MMP-3 is processed into a 53 kDa transient intermediate form by cleaving 35 amino acids of the propeptide. Autolysis of the

intermediate form yields the 45 kDa mature active MMP-3, which may further be processed to a smaller active form of 28 kDa (Okada *et al.* 1986, Freimark *et al.* 1994). Latent and active high molecular forms of MMP-3 can bind to collagen fibrils, and to other ECM components via their C-terminal domains, whereas active low molecular forms do not (Allan *et al.* 1991). MMP-3 in synovia of osteo- and rheumatoid arthtritis is bound either to ECM or is located in cells (Hembry *et al.* 1995). MMP-3 is also bound into the fibrous tissue and osteoid of human bone tissue (Bord *et al.* 1999).

Active MMP-3 is at least capable of degrading cartilage proteoglycans, gelatin, type IV collagen, laminin, fibronectin (Okada *et al.* 1986) and type IX collagen (Okada *et al.* 1989). However, MMP-3 digests fibronectin and gelatin more efficiently at an acidic pH (pH 5.5) (Gunja-Smith *et al.* 1989). MMP-3 is able to remove the NH_2-terminal propeptide from type I procollagen, and in the case of heat denatured type I procollagen also the COOH-terminal propeptide (Okada *et al.* 1986). It also cleaves type III procollagen (Murphy *et al.* 1991). Recombinant human stromelysin-1 acts as a telopeptidase against types II and XI collagens, and hydrolyses collagen type X (Wu *et al.* 1991) and aggrecan (Flannery *et al.* 1992). Furthermore, MMP-3 degrades fibrinogen and fibrin (Bini *et al.* 1996), IL-1β (Ito *et al.* 1996), decorin (Imai *et al.* 1997), urokinase-type plasminogen activator (Ugwu *et al.* 1998) and plasminogen activator inhibitor-1 (PAI-1) (Lijnen *et al.* 2000). ProMMP-3 may contribute to plasminogen activation through complexing with both plasminogen and tissue-type plasminogen activator (t-PA), rather than hydrolysing t-PA (Arza *et al.* 2000). MMP-3 also cleaves serpin α2-antiplasmin (Lijnen *et al.* 2001) and IgG (Gearing *et al.* 2002) and type II procollagen both at the telopeptide and N-proteinase sites (Fukui *et al.* 2002).

The second human stromelysin, MMP-10 (stromelysin-2), was cloned from rheumatoid synovial fibroblasts (Sirum & Brinckerhoff 1989). Other human cells such as keratinocytes (Windsor *et al.* 1993), T-lymphocytes (Conca & Willmroth 1994), chondrocytes, osteoblasts and osteoclasts (Bord *et al.* 1999) also produce MMP-10. The MMP-10 gene contains elements for PMA, EGF and IL-1β induction within its promoter region (Sirum & Brinckerhoff 1989). MMP-10 is transcribed into 1.8 kb mRNA (Conca & Willmroth 1994). Latent MMP-10 is 54 kDa, and after proteolytic activation has a molecular mass of 44 kDa (Windsor *et al.* 1993). MMP-10 is 82% similar to MMP-3 (Sirum *et al.* 1989). Active MMP-10 cleaves collagen types III, IV and V, fibronectin and gelatin (Nicholson *et al.* 1989). It also cleaves proteoglycan in both acid and neutral environments (Fosang *et al.* 1991).

2.8.1.3 Others

Human MMP-12, also called macrophage metalloelastase, is mainly expressed by alveolar macrophages (Shapiro *et al.* 1993). Generally, it is not expressed at detectable levels in normal adult tissues. However, MMP-12 expression is induced as a response to inflammatory stimuli such as endotoxin, or in remodeling tissues like the placenta (Belaaouaj *et al.* 1995). The promoter sequence of MMP-12 contains a TATA-box, AP-1 motif and a PEA3 element (Belaaouaj *et al.* 1995). The 54 kDa proMMP-12 is processed to an active enzyme of 22 kDa, which degrades elastin (Shapiro *et al.* 1993). MMP-12 is

also able to degrade type IV collagen, gelatin, fibronectin, laminin, vitronectin, proteoglycan and myelin basic protein. It cleaves α1-antitrypsin and releases tumour necrosis factor (TNF) from a proTNF fusion protein (Chandler *et al.* 1996). MMP-12 also cleaves tissue factor pathway inhibitor (Belaaouaj *et al.* 2000), fibrinogen and Factor XII (Hiller *et al.* 2000), and urokinase-type plasminogen activator receptor (uPAR) (Koolwijk *et al.* 2001).

MMP-19, cloned from human mammary gland, was at first designated as MMP-18 (Cossins *et al.* 1996). However, *Xenopus* collagenase-4 represents the 18th MMP. Furthermore, it was found that MMP isolated from a liver cDNA library (Pendas *et al.* 1997b), was identical to the mammary gland MMP, and was thus designated as the 19th MMP, MMP-19. MMP-19 has also been isolated from synovial blood vessels of a rheumatic arthritis patient with the name RASI-1 (Kolb *et al.* 1997). MMP-19 differs from other known MMPs in that it has an insertion of acidic amino acids at the hinge region, and two potential glycosylation sites in the hemopexin domain (Cossins *et al.* 1996, Pendas *et al.* 1997b). In addition, proline-94 is replaced with a glutamic acid in the conserved cysteine-switch motif (Cossins *et al.* 1996). MMP-19 is able to associate with the cell surface through its hemopexin domain (Mauch *et al.* 2002). In addition to several internal organs (Pendas *et al.* 1997b), MMP-19 is expressed at the surface of blood mononuclear cells, lymphocytes (Sedlacek *et al.* 1998), blood vessels (Kolb *et al.* 1997, 1999), normal breast tissue, and both malignant and benign breast lesions (Djonov *et al.* 2001), lung fibroblasts and at the myeloid cell surface (Mauch *et al.* 2002).

The MMP19 promoter has several cis-acting regulatory elements similar to other MMPs, including a TATA-box, an AP-1 binding site and a binding site for transcription factors of the Ets family (PEA3 element) (Mueller *et al.* 2000). MMP-19 full lenght cDNA encodes a 508 residue polypeptide with predicted molecular weight of 57 kDa (Cossins *et al.* 1996, Pendas *et al.* 1997b). MMP-19 degrades gelatin (Sedlacek *et al.* 1998), type IV collagen, laminin, nidogen, tenascin-C, fibronectin, type I gelatin (Stracke *et al.* 2000b), cartilage aggrecan and oligomeric matrix protein (COMP) (Stracke *et al.* 2000a).

In mature tissues, MMP-20 (enamelysin) is almost exclusively expressed by odontoblasts, cells capable of forming tooth dentin matrix, and to a lesser extent in pulp tissue (Llano *et al.* 1997, Sulkala *et al.* 2002). During tooth development MMP-20 localizes to ameloblasts, odontoblasts and enamel and to a lesser extent to dentin (Bartlett *et al.* 1996, Den Besten *et al.* 1998, Caterina *et al.* 1999, 2000, Takata *et al.* 2000). Recently, MMP-20 was found to be expressed in oral squamous cell carcinoma (SCC) cells *in vitro* (Väänänen *et al.* 2001), but to date they are the only cells outside the tooth-forming cells that have been shown to express MMP-20, despite various attempts. The MMP-20 transcript utilizes two alternative polyadenylation sites, giving two MMP-20 transcripts of sizes of 2 kb and 4 kb, respectively (Bartlett *et al.* 1996). The open reading frame of the MMP-20 cDNA codes for a 483 amino acid protein. Latent MMP-20 has a molecular weight of 54.4 kDa and the active form is 42.6 kDa (Bartlett *et al.* 1996, Llano *et al.* 1997, Li *et al.* 1999). In addition, 42.6 kDa active forms may be further cleaved into several smaller enzymes ranging from 18 kDa to 38 kDa, possessing differential catalytic activities (Den Besten *et al.* 1998, Ryu *et al.* 1999, Li *et al.* 1999). MMP-20 differs from other MMPs in that it has an insertion of basic residues in the hinge region. The protein sequence does not contain any glycosylation sites (Llano *et al.* 1997). MMP-20 cleaves

amelogenin (Llano *et al.* 1997), gelatin, casein, aggrecan and cartilage oligomeric matrix protein (COMP) (Fukae *et al.* 1998, Ryu *et al.* 1999, Stracke *et al.* 2000a), fibronectin, type IV collagen, laminin-1 and -5 and tenascin-C (Väänänen *et al.* 2001).

MMP-22 (CMMP) has been cloned from chicken embryo fibroblasts (Yang & Kurkinen 1998). A human homologue has not been identified. MMP-22 is a 472 amino acid polypeptide with a unique cysteine in the catalytic domain, which is at the same position in the catalytic domains of both MMP-19 and XMMP. The calculated molecular weight of the active CMMP is 42 kDa. Recombinant MMP-22 digests both gelatin and casein with equal efficiency as recombinant MMP-1. (Yang & Kurkinen 1998.)

The MMP-27 1655 bp cDNA has been cloned by Benoit de Coignac *et al.* (unpublished), and submitted to the nucleotide genbank of NCBI with the accession number of AF195192. It has not yet been further characterized or published.

2.8.2 Gelatin-binding MMPs

This subgroup of metalloproteinases was originally described as type IV collagenases, because of their ability to cleave type IV collagen. One of these, MMP-2 (gelatinase-A, 72 kDa type IV collagenase) was originally purified from highly a metastatic murine tumour (Liotta *et al.* 1981, Salo *et al.* 1985). Since then, it has been found to be expressed in several normal and malignant human tissues. In oral tissues, gingival fibroblasts express MMP-2 (Hipps *et al.* 1991). In human hard tissues, MMP-2 is produced by osteoblasts and odontoblasts (Tjäderhane *et al.* 1998b, Rifas *et al.* 1989). MMP-2 has also been identified in sound (Martin-De Las Heras *et al.* 2000) and in carious (Tjäderhane *et al.* 1998a) human dentin.

The MMP-2 gene codes for a single mRNA of 3.1 kb (Collier *et al.* 1988). The promoter of the human MMP-2 gene has no TATA box or TPA/PMA responsive element (Huhtala *et al.* 1990). Nor does it have a CAAT box, but a binding site for the transcription factor AP-2 exists in the first exon (Huhtala *et al.* 1990). Further MMP-2 sequence analysis has revealed other important transcription factor binding sites in the promoter region, such as AP-1, PEA3, C/EBP, CREB, Ets-1 and Sp1 sites (Qin *et al.* 1999). In addition, the MMP-2 promoter has a binding site for the tumour suppressor and transcription factor, p53 (Bian & Sun 1997). The Sp1 and AP-2 elements have been reported to mediate constitutive human MMP-2 expression (Qin *et al.* 1999), whereas interaction of AP-2 with the transcription factor YB-1 within an enhancer element (RE-1) induces MMP-2 gene transcription (Mertens *et al.* 1998). Further characterization reveals two potential N-linked glycosylation sites at Asn^{546} and Asn^{613}. However, no N-linked oligosaccaharides have been found with the MMP-2 protein (Collier *et al.* 1988). The molecular weight of proMMP-2 is 70 to 72 kDa, and active species are 65 kDa and 62 kDa (Stetler-Stevenson *et al.* 1989b, Hipps *et al.* 1991).

MMP-2 preferentially cleaves gelatin and type IV collagen, but also types V and VII collagens and fibronectin (Collier *et al.* 1988), and type X collagen (Welgus *et al.* 1990). MMP-2 is also able to cleave soluble, triple helical type I collagen at the typical Gly-Ile/Leu sites, producing the ¾ and ¼ fragments (Aimes & Quigley 1995). The catalytic and hemopexin domains of MMP-2, but not the fibronectin domain, are responsible for

collagenolysis (Patterson *et al.* 2001). In addition, MMP-2 degrades cartilage proteoglycan and elastin (Okada *et al.* 1990), IL-1β (Ito *et al.* 1996) decorin (Imai *et al.* 1997) and laminin-5 (Giannelli *et al.* 1997).

MMP-2 exists in the ECM bound to type I and type IV collagen molecules, gelatin and laminin. MMP-2 binds to type I collagen through the fibronectin domain, which stabilises it from autolysis, thereby controlling its activity (Allan *et al.* 1995, Ellerbroek *et al.* 2001). On the other hand, latent MMP-2 is one of the few MMPs so far known to localize to the cell membrane for proteolytic activation (Sato *et al.* 1994).

The second gelatinase MMP-9 (92 kDa type IV collagenase, gelatinase B) is produced in human macrophages and polymorphonuclear leukocytes (Murphy *et al.* 1989). It has also been localized into the endothelial cells and synovial fibroblasts in rheumatoid arthritis synovium (Ahrens *et al.* 1996). MMP-9 is expressed by osteoclasts in the human normal bone tissues, implicating a role in the bone remodeling (Okada Y *et al.* 1995). Mature human intact odontoblasts also express MMP-9 (Tjäderhane *et al.* 1998b). In addition, it has been identified in human dental caries lesion (Tjäderhane *et al.* 1998a) and saliva (Davis 1991). However, MMP-9 is not expressed by human gingival fibroblasts (Bolcato-Bellemin *et al.* 2000). Like MMP-2, MMP-9 may exist in the ECM bound to type I collagen, gelatin or laminin. (Allan *et al.* 1995). Localization of the activated MMP-9 to the cell surface by the hyaluronan receptor CD44 may mediate the activation of latent TGF-β by MMP-9 (Bourguignon *et al.* 1998, Yu & Stamenkovic 2000).

The MMP-9 gene is transcribed into a 2.5 kb mRNA species (Huhtala *et al.* 1991). The 5' flanking region of the gene contains binding sites for AP-1, NF-κB, and Sp1, which synergistically mediate the induction of MMP-9 gene expression by TPA or TNF-α, and TGF-β inhibitor element (TIE) (Huhtala *et al.* 1991, Sato & Seiki 1993). The GT box located downstream of the AP-1 site is essential for the induction of gene transcription by v-Src, which is also able to mediate promoter activation via the AP-1 site (Sato *et al.* 1993). Ets and Sp-1 are essential for activation of MMP-9 gene expression in fibroblasts (Himelstein *et al.* 1998). NF-κB is necessary for the upregulation of MMP-9 gene by inflammatory cytokines, IL-1α or TNF-α, but not by bFGF or PDGF (Bond *et al.* 1998). AP-1 slightly mediates the gene transcription by bFGF, PDGF, IL-1α or TNF-α (Bond *et al.* 1998). Functional polymorphism in the promoter of the MMP-9 gene results in variation in its expression at the transcriptional level (Peters *et al.* 1999).

MMP-9 is synthesized as a 78.4 kDa prepropeptide, and is secreted as a glycosylated 92 kDa proenzyme (Wilhelm *et al.* 1989). Proteolytic activation of the zymogen yields an active MMP-9 enzyme of 82 kDa (Ogata *et al.* 1992). MMP-9 may exist as a monomer, homodimer, or as a complex with lipocalin in neutrophils (Kolkenbrock *et al.* 1996). Active MMP-9 hydrolyses gelatin, native type IV collagen, elastin (Murphy *et al.* 1991), α2 chain of type I collagen, native collagens types III, V, XI and XIV (Okada *et al.* 1992, O'Farrell & Pourmotabbed 1998) and type II procollagen both at the telopeptide and N-proteinase sites (Fukui *et al.* 2002). MMP-9 is also able to cleave the cartilage proteoglycan, aggrecan, although very slowly compared to MMP-2, MMP-3 or MMP-7 (Fosang *et al.* 1992). MMP-9 hydrolyses human plasminogen generating angiostatin fragments (Patterson & Sang 1997), and degrades IL-1β (Ito *et al.* 1996). One unique feature of MMP-9 is that latent MMP-9 (purified from placenta tissue sections) is catalytically active against both the fluorogenic peptide MCA-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 and gelatin substrates (Bannikov *et al.* 2002).

2.8.3 Furin-activated secreted MMPs

MMP-11, or stromelysin-3, was originally characterized from breast carcinoma and has been shown to be 36–40% similar with MMP-3 or MMP-10 or MMP-1 (Basset *et al.* 1990). The promoter of the MMP-11 gene does not contain an AP1 element, which typically exists in this group of several other MMPs, but instead contains a DR1 retinoic acid responsive element (Anglard *et al.* 1995), and is induced by retinoic acid in human fibroblasts (Guerin *et al.* 1997). The MMP-11 promoter contains a TPA-responsive element, required for basal gene expression, and C/EBP-binding site for inducible gene regulation (Luo *et al.* 1999). MMP-11 expression is induced by TGF-β in osteoblasts and fibroblasts (Delany & Canalis 2001).

A recombinant MMP-11 is cleaved to a range of different sizes from 20 to 65 kDa (Pei *et al.* 1994). Of these, the 45 kDa species exhibits catalytic activity capable of cleaving α2-macroglobulin and α1-proteinase inhibitor, which inhibits serine proteinases (Pei *et al.* 1994). MMP-11 is also able to cleave insulin-like growth factor-binding protein-1 (IGFBP-1) indicating a possible role in regulating availability of IGF-1 (Manes *et al.* 1997). No other substrates have been found for MMP-11 to date.

The recently identified MMP-28 (epilysin) cDNA codes for a 520 amino acid protein with molecular mass of 59 kDa (Lohi *et al.* 2001, Marchenko & Strongin 2001). It is expressed in testis, lungs, heart, colon, intestine, brain and skin, fetal kidney, and in several tumour types (Lohi *et al.* 2001, Marchenko & Strongin 2001). The MMP-28 promoter contains Sp1/Sp3 binding GT-box, but does not contain the TATA-box and CAAT sequence typical for most MMPs (Illman *et al.* 2001). MMP-28, capable of degrading at least casein, shares 40% homologue with MMP-19. The cysteine-switch motif of the MMP-28 has a threonine-94 residue instead of proline-94, present in all other MMPs except MMP-19. MMP-28 also has an 11 amino acid insertion after cysteine-switch motif followed by an RRKKR furin recognition site. (Lohi *et al.* 2001, Marchenko & Strongin 2001.)

2.8.4 Vitronectin-like insert MMPs

MMP-21 has been partially cloned from human multi-tissue gene library (Marchenko *et al.* 2001). The 1038 bp fragment encodes a partial sequence of the putative catalytic domain followed by the putative hinge and hemopexin domain. This partial sequence of the human MMP-21 is 73% identical to the *Xenopus* XMMP/MMP-21. *Xenopus* XMMP has 604 amino acids including a putative 22-residue signal peptide (Yang *et al.* 1997). Characteristic of XMMP is that located in its propeptide domain is a 37 amino acid-long insertion domain (ID), related to vitronectin, that has not been found in other MMPs. In addition, it lacks a proline-rich hinge region between the catalytic and hemopexin/ vitronectin-like domain (Yang *et al.* 1997).

2.8.5 Minimal-domain MMPs

MMP-7 is also known as PUMP-1 (putative metalloproteinase) or matrilysin-1. Human MMP-7 cDNA was cloned from tumours (Muller *et al.* 1988). It is expressed in osteoarthritic cartilage (Ohta *et al.* 1998), and is tightly bound into ECM heparin sulfate proteoglycan (Yu & Woessner 2000). The gene promoter of MMP-7 contains TATA, AP-1 and PEA3 elements (Gaire *et al.* 1994). Latent MMP-7 has molecular weight of 28 kDa, which can be activated by APMA into active species of 21 and 19 kDa (Quantin *et al.* 1989). MMP-7 has broad substrate spectrum, and it is capable of degrading several components, some of which also exist in dental tissue. These include fibronectin, gelatin, proteoglycan, decorin, tenascin-C, laminin, osteonectin, osteopontin, E-cadherin, proTNF-α and IgG (Quantin *et al.* 1989, Miyazaki *et al.* 1990, Murphy *et al.* 1991, Gearing *et al.* 1994, 2002, Siri *et al.* 1995, Imai *et al.* 1997, Sasaki *et al.* 1997, Agnihotri *et al.* 2001, Noe *et al.* 2001, Davies *et al.* 2001). MMP-7 also fragments corneal collagen type XVIII NC1 domain, generating 28 kDa endostatin (Lin HC *et al.* 2001), and type II procollagen (Fukui *et al.* 2002).

Minimal domain MMP-26 (matrilysin-2, endometase) was simultaneously cloned from a fetal cDNA library (de Coignac *et al.* 2000), human endometrial tumours (Park *et al.* 2000) and placenta (Uria & Lopez 2000). It has a threonine residue adjacent to the Zn-binding site characteristic of matrilysins (Park *et al.* 2000, Uria & Lopez 2000). MMP-26 shares 39% homology to MMP-7 (Park *et al.* 2000). The MMP-26 gene is located chromosome 11p5 (Uria & Lopez 2000) and is transcribed into an 1.03 kb size mRNA species (Park *et al.* 2000). Latent MMP-26 has a molecular weight of 28–29 kDa and active MMP-26 is 19 kDa (de Coignac *et al.* 2000, Park *et al.* 2000, Uria & Lopez 2000). MMP-26 cleaves gelatin, α1-proteinase inhibitor, TNF-α convering enzyme (Park *et al.* 2000), type IV collagen, fibronectin, fibrinogen (Uria & Lopez 2000), β-casein (de Coignac *et al.* 2000) and vitronectin (Marchenko *et al.* 2001).

ProMMP-26 contains a unique histidine residue instead of arginine residue in the cysteine-switch motif (PH(81)CGXXD) (Park *et al.* 2000, Marchenko *et al.* 2001, Marchenko *et al.* 2002). Since PH(81)CGXXD is not functional, proMMP-26 is not activated by conventional pathway, although His(81)Arg(81) mutation restores functionality to the cysteine-switch motif in the prodomain of MMP-26 (Marchenko *et al.* 2002). Marchenko and colleagues (2002) further show that autolytic LLQ(59)(60)QFH cleavage upstream of the cysteine-switch motif induces the proteolytic activity of recombinant proMMP-26, indicating an alternative activation pathway for this proenzyme.

2.8.6 Type I transmembrane MMPs

MMP-14 (MT1-MMP) was originally discovered as being able to activate proMMP-2 on the cell surface of invasive lung carcinoma cells (Sato *et al.* 1994). In mineralized tissues, MMP-14 is expressed by mouse osteoblasts (Mizutani *et al.* 2001), rabbit osteoclasts (Sato *et al.* 1997), and by odontoblasts and ameloblasts of developing porcine tooth

tissues (Caron *et al.* 1998), and *in vitro* also by human osteoblast-like cells (Luo & Liao 2001). The MMP-14 gene promoter contains several regulatory elements, such as four CCAAT-boxes and one Sp-1 site, but no TATA-box (Lohi *et al.* 2000). MMP-14 mRNA is 4.5 kb and encodes a 582 amino acid protein (Sato *et al.* 1994). The molecular mass for latent MMP-14 is 63–66 kDa and for active MMP-14 is 54 kDa (Sato *et al.* 1994, Sang and Douglas, 1996). The active enzyme may further be processed into an inactive 43 kDa form (Lohi *et al.* 1996). MMP-14 molecules form a multimeric complex with each other through hemopexin and cytoplasmic domains, which mediates its autocatalytic processing and proMMP-2 activation (Itoh *et al.* 2001, Lehti *et al.* 2002). The propeptide domain of MMP-14 may act as an intramolecular chaperone for efficient trafficking of MT1-MMP to the cell surface (Cao *et al.* 2000, Pavlaki *et al.* 2002). MMP-14 has two potential proprotein convertase recognition motifs, RRPR/RRKR, for possible intracellular furin activation of the zymogen (Sato *et al.* 1994, Will & Hinzmann 1995, Yana & Weiss 2000).

Native and transmembrane deleted MMP-14 cleaves types I, II and III collagens (d'Ortho *et al.* 1997, Ohuchi *et al.* 1997). Soluble MMP-14 also cleaves gelatin, cartilage proteoglycan, fibronectin, vitronectin, tenascin, nidogen, aggrecan, perlecan, laminin-1, α1-proteinase inhibitor and α2-macroglobulin (Pei & Weiss 1996, d'Ortho *et al.* 1997, Ohuchi *et al.* 1997) and type II procollagen both at the telopeptide and N-proteinase sites (Fukui *et al.* 2002). MMP-14 is able to cleave fibrinogen and also inactivate Factor XII (Hiller *et al.* 2000). It degrades the hyaluronan receptor CD44 (Kajita *et al.* 2001), the receptor of complement component (gC1qR) (Rozanov *et al.* 2001) and the cell surface adhesion receptor, tissue transglutaminase (tTG) (Belkin *et al.* 2001). MT1-MMP is capable of processing pro-αV integrin, thus exhibiting integrin convertase activity (Deryugina *et al.* 2001, 2002a, Ratnikov *et al.* 2002). MMP-14 complexed to TIMP-2 possesses gelatinolytic activity (Imai *et al.* 1996). MMP-14 on the cell membrane is able to degrade type I collagen more efficiently after addition of proMMP-2 (Atkinson *et al.* 2001).

MMP-14 may itself act as a regulatory protein since the cytoplasmic domain of MMP-14 activates extracellular signal-regulated protein kinase (ERK), indicating a role in a signal transduction pathway involved in cell migration or gene regulation (Gingras *et al.* 2001). MMP-14 up-regulates vascular endothelial growth factor (VEGF) in human glioma U251 xenografts in athymic mice (Deryugina *et al.* 2002b).

The MMP-15 (MT2-MMP) 3.6 kb transcript was originally characterised in human lung and shown to be expressed by several other internal organs (Will & Hinzmann 1995). MMP-15 is 75.8 kDa in size, containing a possible glycosylation site at N150. It is 73.9% similar with MMP-14 (Will & Hinzmann 1995). Recombinant catalytic domain of MMP-15 cleaves fibronectin, tenascin, nidogen, aggrecan, perlecan and laminin (d'Ortho *et al.* 1997). MMP-15 degrades the cell surface adhesion receptor, tissue transglutaminase (tTG) (Belkin *et al.* 2001).

MMP-16 (MT3-MMP) was cloned from a placenta cDNA library (Takino *et al.* 1995). It is also expressed in the brain at high levels. The MMP-16 transcript is 12 kb in size, encoding a polypeptide of 604 amino acids (Takino *et al.* 1995). ProMMP-16 molecular mass is 64 kDa. Smaller, possibly active forms of 52, 33 and 30 kDa have also been characterised. Soluble MMP-16 is obtained by an alternative mRNA splicing (Matsumoto *et al.* 1997). MMP-16 also has a RXKR motif for furin processing. Its catalytic domain

shares 66% homology with MMP-14 (Takino *et al.* 1995), and it is also capable of activating proMMP-2 (Takino *et al.* 1995). Soluble MMP-16 hydrolyses type III collagen, α2(I) collagen chain, cartilage proteoglycan, gelatin, fibronectin, vitronectin, laminin-1, transferrin, α1-proteinase inhibitor and α2-macroglobulin (Matsumoto *et al.* 1997, Shimada *et al.* 1999). MMP-16 also degrades the cell surface adhesion receptor, tissue transglutaminase (tTG) (Belkin *et al.* 2001).

Human MMP-24 (MT5-MMP), encoding a 645 amino acid polypeptide, is expressed in the brain, kidney, pancreas and lung. The cleavage site for shedding of MMP-24 is located at (545) RRKERR, where a furin recognition site also exists, and therefore proteolytic activity of MMP-24 could be regulated by shedding with furin-type convertase (Wang & Pei 2001). However, the substrates for MMP-24 remain largely unknown.

2.8.7 GPI-linked MMPs

MMP-17 (MT4-MMP) is widely expressed in the internal organs and by leukocytes (Puente *et al.* 1996). Two transcripts of 2.7 kb and 7.5 kb are obtained, which are possibly the result of alternative use of distinct polyadenylation sites (Puente *et al.* 1996). MMP-17 has a furin processing recognition motif, RXR/KR, within its structure (Puente *et al.* 1996). Further characterization has revealed that functional MMP-17 is translated into proteins of 67 and 71 kDa sizes (Kajita *et al.* 1999). Human MMP-17 is able to cleave gelatin, but not other typical ECM components (Kolkenbrock *et al.* 1999, Wang *et al.* 1999), whereas mouse MMP-17 exhibits the capability of hydrolysing pro-tumour necrosis factor α (TNF-α), fibrinogen and fibrin (English *et al.* 2000), suggesting differences in functions between species.

Human MMP-25 (MT6-MMP, leukolysin) is a 562 amino acid polypeptide expressed by leukocytes and neutrophils, and in lung, spleen and brain tumours (Velasco *et al.* 2000, Kang *et al.* 2001). PMA or IL-8 induces release of MMP-25 form neutrophils as a soluble enzyme with molecular size of 56 kDa and minor forms ranging from 38 to 45 kDa (Kang *et al.* 2001). MMP-25 degrades type IV collagen, gelatin, fibronectin and fibrin (English *et al.* 2001).

2.8.8 Type II transmembrane MMPs

MMP-21 and MMP-22 genes were found to be identical to each other. These genes are located on chromosome 1p36.3, which was duplicated during evolution yielding two identical enzymes (Gururajan *et al.* 1998a, 1998b). Currently, NCBI has named MMP-21 as MMP-23A and MMP-22 as MMP-23B. MMP-23, cloned by Velasco and colleagues (1999), is identical with MMP-23B. A characteristic feature of these genes is that instead of a consensus cysteine-switch motif, they have a sequence ALCLLPA containing a single conserved cysteine for maintaining the enzyme latency. The open reading frame of

MMP-23B codes for a protein of 390 amino acids with a molecular mass of 43.9 kDa (Velasco *et al.* 1999). The MMP-23B protein contains four potential N-glycosylation sites. Structural analysis of the mouse counterpart of MMP-23 has revealed that it has an N-terminal signal anchor targeting it to the cell membrane (Pei *et al.* 2000). MMP-23 also has cysteine array and immunoglobulin (Ig)-like domains in its C-terminal end (Pei *et al.* 2000). Several transcripts are expressed due to alternative splicing of MMP-23A and MMP-23B, and these are expressed in heart, placenta, ovary, testis and prostate (Gururajan *et al.* 1998a, Velasco *et al.* 1999).

2.9 Transcriptional regulation of MMPs

Since MMPs have the fundamental ability to degrade virtually all types of matrix components, they are very precisely regulated at the transcriptional level. Most of the MMPs are inducibly transcribed, whereas only some are constitutively expressed. Since members of the TGF-β superfamily are suggested to be associated with healing reactions of teeth after injury, detailed overview of the apparent actions of TGF-β1 and BMP-2 on MMPs is relevant.

2.9.1 Signal transduction by TGF-βs and BMPs

TGF-β is stored in the ECM as a large latent complex composed of TGF-β, its propeptide TGF-β latency-associated protein (LAP), and a latent TGF-β-binding protein (LTBP) (Taipale *et al.* 1994). However, different inactive TGF-β forms may exist, since osteoblast-like cells produce small latent TGF-β complex lacking the LTBP (Dallas *et al.* 1994). The matrix association and release of TGF-β form a finely regulated network for the maintenance of ECM. Multiple proteases such as serine proteases or MMPs are able to release TGF-β from ECM, and further proteolytic activation of the inactive TGF-β by MMPs or by acid treatment, enables its signalling through type I and type II serine/ threonine kinase receptors (Brown *et al.* 1990, Taipale *et al.* 1992, Wrana *et al.* 1994, Yu & Stamenkovic 2000, Maeda *et al.* 2002). Similarly, BMPs mediate their signal through specific type I and type II serine/threonine kinase receptors (Liu *et al.* 1995).

Co-operation of type I and type II receptors leads to downstream phosphorylation of SMAD proteins, a family of cytoplasmic signal transducer proteins (Hoodless *et al.* 1996, Nakao *et al.* 1997b), which localise to the nucleus, where they bind to DNA and affect gene transcription. (See Fig. 3) TGF-β may induce c-Fos and c-Jun proto-oncogenes, which heterodimerize to form an AP-1 complex (Risse *et al.* 1989, Subramaniam *et al.* 1995). For many TGF-β regulated genes, SMADs co-operate with the AP-1 complex at the AP-1 binding site, although SMADs may also independently bind to AP-1 sequence, to regulate transcription of genes such as MMP-1 or MMP-13 (Zhang *et al.* 1998, Yuan & Varga 2001, Tardif *et al.* 2001). Inhibitory SMADs act as negative regulators of signalling by the TGF-βs or BMPs (Nakao *et al.* 1997a, Imamura *et al.* 1997).

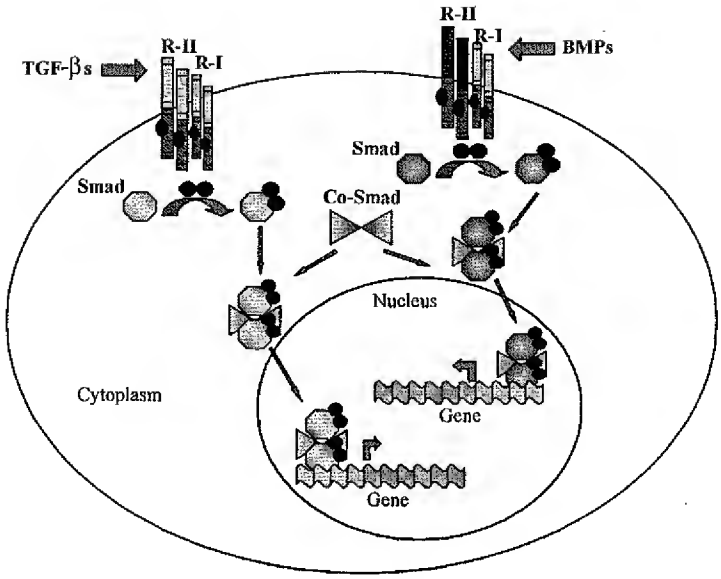

Fig. 3. Signalling cascades of TGF-β and BMP. (Modified from Miyazono *et al.* 2001).

2.9.2 Regulation of MMPs by TGF-β1 or BMP-2

TGF-β1 induces expression of both MMP-11 and MMP-13 in osteoblasts and fibroblasts (Uría *et al.* 1998, Jiménez *et al.* 1999, Delany & Canalis 2001), MMP-10 in keratinocytes (Madlener *et al.* 1996, Rechardt *et al.* 2000), and MMP-2 in gingival fibroblasts (Overall *et al.* 1991). However, MMP-2 induction may be dose-dependent or dependent on the fibroblasts passage and type, since Salo and colleagues (1991) have not seen significant stimulation of the level of MMP-2 expression in gingival fibroblasts, and Chang and colleagues (2001), on the other hand, report that TGF-β1 decreases MMP-2 in pulp cells. TGF-β1 up-regulates MMP-9 expression in odontoblasts, osteoblasts, normal equine chondrocytes and oral mucosal keratinocytes (Salo *et al.* 1991, Tjäderhane *et al.* 1998b, Festuccia *et al.* 2000, Thompson *et al.* 2001) but not significantly in gingival fibroblasts (Salo *et al.* 1991). On the other hand, TGF-β suppresses TNF-α induced MMP-9 secretion in monocytes (Vaday *et al.* 2001). In addition, MMP-1, MMP-3 and MMP-8 are down-regulated by TGF-β in chondrocytes (Shlopov *et al.* 1999, Wang *et al.* 2002). TGF-β1 also suppresses cytokine-induced (*e.g.* IL-1α) MMP-1 expression in pulp fibroblasts and MMP-12 expression in macrophages (Tamura *et al.* 1996, Feinberg *et al.* 2000). MMP-1 inhibition is mediated through cellular Smad3 and Smad4, and thus Smad mediated repression of the MMP-1 expression may be important for preventing excessive matrix degradation induced by inflammatory cytokines (Yuan & Varga 2001). On the other hand, TGF-β does not affect on MMP-20 expression in tongue carcinoma (SCC) cells (Väänänen *et al.* 2001).

There is little data on the regulatory effect of BMP-2 on the MMPs to date. BMP-2 has been shown to inhibit MMP-1 expression in adult human osteoblasts, and also MMP-13 expression in chondrocytes during fetal bone development (Takiguchi *et al.* 1998, Johansson *et al.* 1997). Even though there are no data available on the synergistic effect of TGF-β1 and BMP-2 on MMPs, evidence suggests that signalling cascades of the TGF-β and BMP are connected to each other. It has been shown that BMP may mimic or oppose the effects of TGF-β in bone and bone cell culture models (Centrella *et al.* 1995). For example, in human bone marrow stromal cells co-treatment with BMP-2 and TGF-β2 reduces the TGF-β2-enhanced DNA and type I collagen synthesis (Fromigué *et al.* 1998). In addition, TGF-β1 has been shown to inhibit the expression of BMP-2 in rat osteoblasts, but to stimulate its expression in human pulp cells (Harris *et al.* 1994, Calland *et al.* 1997). In human bone marrow, TGF-β diminishes the stimulatory effect of BMP-2 on osteocalcin levels, and alkaline phosphatase activity and mRNA levels (Fromigué *et al.* 1998).

2.10 Activation of proMMPs

Another level at which to control ECM proteolysis is the activation of latent zymogen in several biological processes including physiological tissue remodeling of bone and cartilage, and in several pathological conditions. A majority of the MMPs are secreted as latent zymogens, achieving catalytic properties in the ECM milieu. Some MMPs are intracellularly processed, whereas a few are processed at the cell membrane, into fully active enzymes.

Non-proteolytic compounds such as organomercurial chemicals (4-aminophenyl-mercuric acetate (APMA)), denaturants (SDS) and conformational perturbants (detergents) or proteases may open the cysteine to zinc switch to trigger proMMP activation. Changes in pH also affect zymogen activation, since an acidic condition followed by neutralization has been shown to activate at least proMMP-9 (Davis 1991, Tjäderhane *et al.* 1998a).

The prodomain of MMP is removed in an autocatalytical manner or by proteases, and the cysteine is replaced by a water molecule to allow enzyme catalysis to proceed (Springman *et al.* 1990, Van Wart & Birkedal-Hansen 1990). For example, the 43 kDa proMMP-1 activation by APMA leads first to a weakly active MMP-1, which is converted to the 41 kDa fully active form by another MMP, MMP-3 (Nagase *et al.* 1992). Autoactivation is a significant mechanism to generate mature and active enzymes for a subset of MMPs (Stricklin *et al.* 1983, Weiss *et al.* 1985, Okada Y *et al.* 1988, Stetler-Stevenson *et al.* 1989b, Wilhelm *et al.* 1989, Freije *et al.* 1994).

MMP-11, MMP-28 and MT-MMPs contain a conserved **RXRXKR** furin-like enzyme recognition motif between their pro- and catalytic domains and, thus, can be activated intracellularly in the Golgi network (Pei & Weiss 1995, Puente *et al.* 1996, Sato *et al.* 1996, Lohi *et al.* 2001, Marchenko & Strongin 2001, Kang *et al.* 2002). However, membrane-anchored proMT1-MMP can be processed into the active enzyme also by a furin-independent route (Yana & Weiss 2000).

Other, already active MMPs or several serine proteases may activate latent proMMPs at the cell surface or pericellularly. Whereas proMMP-2 can be activated by mercurial compounds (Stetler-Stevenson *et al.* 1989b), several physiological endopeptidases such as serine proteases cannot (Okada & Nakanishi 1989). The well described proMMP-2 activation proceeds at the cell membrane through an MT-MMP mediated cascade (Sato *et al.* 1994). In addition, new data indicates that PMN-derived elastase, cathepsin G and proteinase-3 are able to activate proMMP-2 through an MT1-MMP dependent route (Shamamian *et al.* 2001). Essentially, the MT1-MMP mediated proMMP-2 activation includes the formation of MT1-MMP and TIMP-2 complex, serving as a receptor for proMMP-2 binding (Sato *et al.* 1994, Strongin *et al.* 1995, Imai *et al.* 1996, Butler *et al.* 1998, Zucker *et al.* 1998, Fernandez-Catalan *et al.* 1998). The negatively charged C-terminal domain of TIMP-2 facilitates noninhibitory binding to the proMMP-2 C-terminal domain via electrostatic interactions to allow an adjacent free, active MT1-MMP to process proMMP-2 into an active enzyme (Atkinson *et al.* 1995, Strongin *et al.* 1995, Butler *et al.* 1998, Jo *et al.* 2000). (See Fig. 4) TIMP-2 mediates proMMP-2 activation in a dose-dependent manner. ProMMP-2 is processed at low TIMP-2 concentrations, but inhibited at higher concentrations (Kinoshita *et al.* 1998). MT2-MMP may activate proMMP-2 through a TIMP-2 independent route (Morrison *et al.* 2001). Also, other MT-MMPs process proMMP-2 (Takino *et al.* 1995, Llano *et al.* 1999, Velasco *et al.* 2000). However, inconsistent information on human MT4-MMP activation of proMMP-2 exists compared to its mouse counterpart, which does not activate proMMP-2 (Kolkenbrock *et al.* 1999, Wang *et al.* 1999, English *et al.* 2000). It has been shown that activated protein C may activate proMMP-2 independently of the MT-MMP mediated activation cascade (Nguyen *et al.* 2000).

MMP-1, MMP-2 and MMP-26 are able to activate proMMP-9 (Fridman *et al.* 1995, Sang *et al.* 1995, Uria & Lopez 2000). MMP-2 in concert with MT1-MMP can activate proMMP-13 (Knäuper *et al.* 1996c). MMP-3 activates proMMP-1, proMMP-8, proMMP-9 and proMMP-13 (Ito & Nagase 1988, Suzuki *et al.* 1990, Ogata *et al.* 1992, Knäuper *et al.* 1993, 1996a). MMP-7 activates proMMP-1, proMMP-2, proMMP-9 (Quantin *et al.* 1989, Crabbe *et al.* 1994, Sang *et al.* 1995). MMP-7 also activates proMMP-9 bound to TIMP-1, and transiently enhances the gelatinolytic activity of proMMP-2 in a complex with TIMP-2 (von Bredow *et al.* 1998). MMP-10 can activate proMMPs, such as MMP-1, MMP-7, MMP-8 and MMP-9 (Nicholson *et al.* 1989, Knäuper *et al.* 1996b, Nakamura *et al.* 1998). (See Fig. 4) Furthermore, several serine proteases may activate latent MMPs to catalytically active enzymes. These proMMPs include at least MMP-1, MMP-3 and MMP-9 (He *et al.* 1989, Okada & Nakanishi 1989, Okada *et al.* 1992). Other serine proteinases, such as trypsin-2, activate proMMP-2 and proMMP-9 (Sorsa *et al.* 1997).

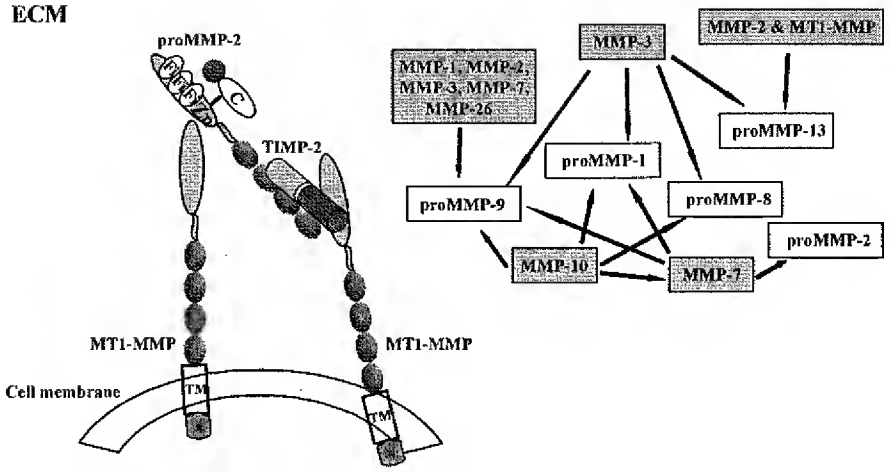

Fig. 4. Some activation routes of latent MMPs.

2.11 Inhibition of MMP activities

The third level of restricting the proteolytic activities of MMPs includes endogenous tissue inhibitors of MMPs (TIMPs). TIMPs specifically inhibit active forms of MMPs, and in some cases, latent MMPs as well, and disturbance in this balance may lead to pathological situations in tissues. Active MMPs may also be inactivated by α-macroglobulins, particularly α2-macroglobulin (Sottrup-Jensen 1989, Sottrup-Jensen *et al.* 1989). Recent findings indicate that serine proteinase inibitor, tissue factor pathway inhibitor-2 (TFPI-2), inhibits MMP-1, MMP-2, MMP-9 and MMP-13 (Herman *et al.* 2001). Calcium-binding proteoglycans (N-Tes, testican-1 or testican-3) are able to inhibit MT1- or MT3-MMP mediated proMMP-2 activation (Nakada *et al.* 2001). In addition, there are many exogenous inhibitors. Some examples include flavonols in green tea such as epigallocatechin-3-gallate or catechins, which inhibit MMP-2, MMP-9, MMP-12 activities and proMMP-2 activation (Demeule *et al.* 2000, Garbisa *et al.* 2001). Several synthetic inhibitors are also good inhibitors of MMPs activities (Mix *et al.* 2001, Bernardo *et al.* 2002). However, this thesis will focus only on the endogenous tissue inhibitors of MMPs.

2.11.1 Tissue inhibitor of matrix metalloproteinase-1, -2, -3 and -4 (TIMP-1, TIMP-2, TIMP-3 and TIMP-4)

An essential feature of all TIMPs is that they have 12 conserved cysteine residues, with conserved relative spacing, and the presence of a 23 to 29 amino acid leader sequence, which is cleaved to produce a mature protein. Crystal structures for TIMPs, and MMP-

TIMP complexes such as TIMP-1 in complex with MMP-3 and TIMP-2 with MT1-MMP have been described (Gomis-Ruth *et al.* 1997, Fernandez-Catalan *et al.* 1998). TIMPs have the shape of an elongated, contiguous wedge consisting of the N-terminal and the C-terminal halves of the polypeptide chains opposing each other (Gomis-Ruth *et al.* 1997). In complexes with MMPs, TIMPs bind with their edge into the entire length of the active-site cleft of MMPs (Fernandez-Catalan *et al.* 1998, Gomis-Ruth *et al.* 1997).

TIMP-1 protein is a 184 amino acid glycoprotein with a molecular mass of 28.5 kDa (Carmichael *et al.* 1986). It contains two possible N-glycosylation sites. The TIMP-1 promoter contains 10 Sp1, six AP-1, six PEA3, 12 AP-2 sites and five CCAAT boxes, in addition to a putative binding site for the transcription factor leader-binding protein 1 (LBP-1) (Clark *et al.* 1997). The upstream TIMP-1 element-1 (UTE-1) is also essential for TIMP-1 transcription (Trim *et al.* 2000). The promoter contains two novel repressive elements, and an unidentified Ets-related factor to suppress transcription (Dean *et al.* 2000). TIMP-1 protein has been detected in human dentin and cementum (Ishiguro *et al.* 1994). In addition, human osteoblasts secrete TIMP-1 constitutively (Rifas *et al.* 1989).

TIMP-2 is a nonglycosylated 194 amino acid protein of 21 kDa molecular mass (Stetler-Stevenson *et al.* 1989a, Boone *et al.* 1990). It has an extended negatively charged C-terminus (Boone *et al.* 1990). The TIMP-2 promoter contains several regulatory elements including five Sp1, two AP-2, one AP-1 and three PEA-3 binding sites (De Clerck *et al.* 1994, Hammani *et al.* 1996). TIMP-2 is transcribed into two mRNAs of 1.2 and 3.8 kb (Hammani *et al.* 1996). Human osteoblasts and chondrocytes secrete TIMP-2 (Rifas *et al.* 1994, Zafarullah *et al.* 1996).

The TIMP-3 polypeptide sequence is 37% and 42% similar to the sequences of TIMP-1 and TIMP-2, respectively (Apte *et al.* 1994). The TIMP-3 protein has 188 amino acids (Apte *et al.* 1994). It has a conserved glycosylation site near the C-terminus. Characterisation of the human recombinant TIMP-3 reveals that it has both a 27 kDa glycosylated and a 24 kDa unglycosylated species (Apte *et al.* 1995). TIMP-3 is localised to the ECM in both its glycosylated and unglycosylated forms (Langton *et al.* 1998). The TIMP-3 gene has four Sp1 sites, but no TATA-box in the promoter (Apte *et al.* 1994, Wick *et al.* 1995). Three TIMP-3 mRNA species of 2.4, 2.8 and 5.5 kb are transcribed from the gene (Apte *et al.* 1994), and are constitutively expressed by human chondrocytes (Su *et al.* 1996).

TIMP-4 is a 195 amino acid polypeptide with molecular mass of 22 kDa (Greene *et al.* 1996). The TIMP-4 polypeptide is 37% identical to TIMP-1 and 51% identical to TIMP-2 and –3 (Greene *et al.* 1996). TIMP-4 is the most neutral TIMP protein under physiological conditions (pH 7.4), having an isoelectric point of 7.34, compared with values of 8.00, 6.45 and 9.04 for human TIMP-1, TIMP-2 and TIMP-3, respectively (Wilde *et al.* 1994, Greene *et al.* 1996). The TIMP-4 gene is transcribed into 1.4 kb mRNA species (Olson *et al.* 1998). Of the calcified tissues, TIMP-4 has been detected in human cartilage (Huang *et al.* 2002).

Limited data exists on effects of TGF-β superfamily members on TIMPs in calcified tissue. It has been shown that TGF-β1 increases the mRNA of both TIMP-1 and TIMP-3 in human chondrocytes and rat osteoblasts (Overall 1995, Su *et al.* 1996, Wang *et al.* 2002), and that BMP-2 stimulates TIMP-1 and TIMP-3 but not TIMP-2 mRNA expression in rat osteoblasts (Varghese & Canalis 1997).

2.11.2 Inhibition of MMPs by TIMPs

Each TIMP binds with both a different rate of interaction and affinity to a target MMP, usually in 1:1 or 2:2 stoichiometrical fashions. TIMP-1 inhibits MMP-1, MMP-3 and MMP-9 more effectively than TIMP-2 (Howard *et al.* 1991, Baragi *et al.* 1994, O'Connell *et al.* 1994, Nguyen *et al.* 1994). TIMP-2 inhibits proMMP-2 over 10-fold more effectively than TIMP-1 (Stetler-Stevenson *et al.* 1989a, Howard *et al.* 1991). However, TIMP-2 has a bi-functional effect on MMP-2 since MT-MMP mediated proMMP-2 activation requires a tiny amount of TIMP-2 to make activation progress, whereas a greater concentration of TIMP-2 inhibits MMP-2 (Kinoshita *et al.* 1998). TIMP-3 inhibits at least MMP-2 and MMP-9 (Butler *et al.* 1999), whereas TIMP-4 is a good inhibitor for all classes of MMPs without remarkable preference for specific MMPs (Stratmann *et al.* 2001). TIMP-4 regulates MMP-2 activity both by inhibiting MT1-MMP and by inhibiting activated MMP-2 (Bigg *et al.* 2001, Hernandez-Barrantes *et al.* 2001).

While TIMPs usually inhibit already active MMP, gelatin-binding MMPs are an exception, since TIMP reversibly binds to the proforms of both MMP-2 and MMP-9. Later, TIMP may be dissociated from the complex, and proMMP activation is allowed to proceed. For example, TIMP-1 binds to the proMMP-9, and further proMMP-9 activation by MMP-3 is prevented until TIMP-1 is inactivated in the complex, *e.g.* by neutrophil elastase, which does not destruct proMMP-9 (Goldberg *et al.* 1992, Itoh & Nagase 1995). However, TIMP-2 may also inhibit the active form of MMP-9 (Olson *et al.* 2000).

Active MMP-13 is an example of an MMP, which is inhibited by all types of TIMPs (Knäuper *et al.* 1996a, Stratmann *et al.* 2001), and MMP-19 is inhibited by all TIMPs, except TIMP-1 (Stracke *et al.* 2000b). The activity of soluble MMP-16 is inhibited by TIMP-2 and TIMP-3, but not TIMP-1 (Shimada *et al.* 1999).

2.12 Suggested roles for MMPs and TIMPs in dentin-pulp complex

MMP-mediated proteolytic processing has several possible roles in dental tissue. MMPs together with their inhibitors may either act in the process of synthesis and mineralization of dentin matrix or, on the other hand, some of the MMPs may be responsible for tissue destruction in pathological injuries such as caries, attrition or pulpal inflammation.

2.12.1 MMPs and TIMPs in dentinogenesis

MMP-3 has been identified in predentin of a rat incisor, at the junction between a proximal and central zone of the predentin (Hall *et al.* 1999). Since MMP-3 is able to hydrolyze proteoglycans, it is highly possible that it may modify proteoglycans, and possibly also other ECM components, during collagen organic matrix organization and mineralization. Further evidence that MMPs may contribute to the dental matrix synthesis comes from studies where MMP-2 has been shown to be transiently secreted by secretory

odontoblasts during tooth development (Sahlberg *et al.* 1992, Heikinheimo & Salo 1995). The expression of MMP-2 increases when odontoblasts secrete primary predentin matrix (mantle dentin), but decreases right after mineralization begins (Sahlberg *et al.* 1992). In addition, mature human odontoblasts secrete MMP-2 and active MMP-2 has been detected in human dentin, giving further indirect evidence that MMPs may play a role in dentin matrix modeling either before or during mineralization (Tjäderhane *et al.* 1998b, Martin-De Las Heras *et al.* 2000). Also, TIMPs may participate in the regulation of dentin formation, since TIMP-1 is shown to be secreted into a predentin (Hoshino *et al.* 1986, Ishiguro *et al.* 1994). Even though these findings do not evidence the exact function of MMPs and TIMPs in dentinogenesis, it is likely that they have a role during dentinogenesis.

2.12.2 MMPs and caries pathobiology

Since latent collagenous activity (Dayan *et al.* 1983, Dumas *et al.* 1985), MMP-2 (Maritn-De Las Heras *et al.* 2000) and MMP-20 (Sulkala *et al.* 2002) have been detected in human dentin, and also MMP-2, MMP-8 and MMP-9 are observed in demineralised caries lesions (Tjäderhane *et al.* 1998a), it has been suggested that host MMPs may degrade collagen matrix in dental caries progression (Tjäderhane *et al.* 1998a). This is further supported by the activation of latent MMPs at acidic environment followed by neutralisation, a reaction comparable to that taking place in caries pathobiology (Tjäderhane *et al.* 1998a), and that acid treated dentin collagen is degraded by endogenous collagenase (Dung *et al.* 1995). Indeed, recent *in vivo* experimens indicate that MMP inhibition may reduce the dentinal caries progression rate (Sulkala *et al.* 2001).

2.12.3 MMPs and pulpitis

Proteolytic enzymes, such as MMPs, are associated, in addition to physiological tissue remodeling, with tissue destruction in inflammatory diseases. Based on findings that inflammatory cytokines, such as interleukin-1-α or -β, regulate the expression of MMPs, especially inducing the expression of MMP-1, MMP-2 and MMP-3 either in dental pulps or pulpal fibroblasts, it is likely that MMPs play a role in the tissue destruction of inflamed dental pulp (Panagakos *et al.* 1996, Tamura *et al.* 1996, O'Boskey & Panagakos 1998, Chang *et al.* 2001, Lin SK *et al.* 2001, Shin *et al.* 2002).

3 Aims of the present study

The biological events in teeth have been actively investigated, but the regulation of dentin formation in both physiological and pathological conditions is still not completely clear. In addition, most of the studies on dentin-pulp complex have been performed in animal models, especially with rats. However, rodent tooth metabolism may differ greatly from that of human, since rodents have continuously growing incisors, and therefore the composition of extracellular matrix of the developing tooth is different from that of the mature tooth. Thus, it is difficult, if not even impossible, to draw any definitive conclusions about the situation in mature human tooth from results obtained from animal models.

Type I collagen is the main protein in the dentin matrix, and it is a key factor in dentin formation and mineralization, although there is evidence that odontoblasts are capable of synthesizing other collagens too, including type III collagen. Roles for MMPs and TIMPs in physiological dental tissue remodeling have also been suggested. However, little is known about the existence of MMPs and TIMPs in mature human teeth to date. Growth factors, such as TGF-β and BMP-2, have the ability to regulate responses of the dentin-pulp complex to external injury, but there are differing views about the stimulatory affect of TGF-β on dentin matrix synthesis and on odontoblast secretory behaviour. In addition, very little is known about the effect of growth factors on MMP or TIMP expression in dentin-pulp complex. Based on this background, the present work was set up with the following aims:

1. to investigate the effect of TGF-β1 on type I and III collagens synthesis in sound, mature human odontoblasts and pulp tissue,
2. to analyze the expression profile of MMPs and TIMPs in mature human odontoblasts and pulp tissue, and
3. to investigate the effects of TGF-β1 and BMP-2 on MMP and TIMP expression, with the use of human odontoblast and pulp tissue culture methods.

4 Materials and methods

More detailed descriptions of the materials and methods are presented in the original publications referred to by their Roman numerals.

4.1 Collection of the samples

All the samples were collected during normal treatment of a patient with her/his informed consent. Intact third molars were received from the University Student Health Care Centre and the Department of Oral and Maxillofacial Surgery, University of Oulu, and were prepared immediately either for use as native samples (uncultured) or for cultures. The biopsies of healthy gingiva were taken during extractions of maxillary canines, and trabecular alveolar bone of maxilla or mandibula from patients undergoing impacted wisdom teeth operations. Bone marrow samples were obtained from patients being operated on for orthopedic reasons as described (Hanemaaijer *et al.* 1997).

4.2 Odontoblasts and pulp tissue (I–IV)

4.2.1 Native samples (I–IV)

Tooth was cut longitudally exposing the pulp chamber. Pulp tissue was removed from a pulp chamber and odontoblasts, remaining in the chamber wall, were scraped off. Pulp tissue and odontoblasts were stored separately either into a Trizol -solution (GibcoBRL, Life Technologies) for use as native samples for mRNA studies, or 1 x Laemmli buffer for protein analyses.

4.2.2 Odontoblast and pulp tissue cultures (I–IV)

The detailed procedures for cultures have been described by Tjäderhane and colleagues (1998b). Briefly, after cutting of the root, the pulp tissue was gently removed with forceps. The crown, with the odontoblasts in the pulp chamber wall, was placed in the Agarose gel pulp chamber facing upward. The pulp chamber was filled with serum-free OPTI-MEM I Reduced Serum Medium (GibcoBRL, Life Technologies), supplemented with appropriate antibiotic-antimycotic solution and 50 µg of ascorbic acid (Sigma). The pulp tissues were cultured separately, with the same medium. The culturing took place at 37°C in the presence of 5% CO_2.

Odontoblasts and pulp tissue were treated with or without TGF-β1, BMP-2 or both together, for 24 h (III, IV), 48 h (I, II) or up to ten days (I) in the serum-free culture medium. The concentration of TGF-β1 was either 1 ng/ml (III, IV) or 10 ng/ml (I, II) and for BMP-2 100 ng/ml (III, IV). After incubation, the cells were used for mRNA studies and medium was collected for protein analysis.

4.3 Other cells (I, II)

4.3.1 Pulp and gingival fibroblasts (I, II)

Pulp and gingiva tissue were cut into small pieces and the tissue pieces were allowed to attach to cell culture clusters. The DMEM medium, supplemented with 10% fetal bovine serum (FBS), was added and the tissue explants were cultured as described above. When the outgrowth of fibroblasts was observed, the tissue pieces were removed. After reaching confluence, the cells were subcultured through several passages. The pulpal (I, II) and gingival fibroblasts (I) were treated with or without 10 ng/ml TGF-β1 for 24 h either in serum-free DMEM or DMEM with 10% FBS. After incubation the medium was collected and total RNA was isolated from the cells.

4.3.2 Osteoblasts (I)

The detailed procedure for establishing osteoblast cell lines has been described by Robey & Termine (1985). Briefly, trabecular alveolar bone was cut into pieces and incubated with DMEM medium supplemented with Ham's Nutrient Mixture F-12 (1:1) for 24 h. After that the pieces were incubated with 1 mg/ml bacterial type IV collagenase with shaking. After 2 h the pieces were cultured with media containing 10% newborn calf serum, 100 µg/ml penicillin G and 50 µg/ml ascorbic acid. After the cells had reached confluence, the bone pieces were removed and the cells trypsinized and divided into three plates (passage 1). The osteoblastic nature of the passage 2 cells was analysed histochemically by determining the alkaline phosphatase production with a leukocyte alkaline phosphatase kit (Sigma). To obtain mRNA for quantitative PCR analysis, the

passage 6 cells were grown to subconfluence, washed with phosphate-buffered saline (PBS) and incubated in serum- and penicillin-free medium containing 0.1% bovine serum album for 48 h. After that, total RNA was isolated from the cells.

4.4 Analysis of mRNA expression

4.4.1 RT-PCR procedure (I–IV)

Total RNA was isolated according to the manufacturer's procedure of Trizol® method (GibcoBRL, Life Technologies). The first strand cDNA was synthesised from 0.2–1 µg of total RNA using 200 units of Superscript™ II RnaseH⁻ Reverse Transcriptase (GibcoBRL, Life Technologies) and random hexamer primers. cDNA was amplified with 1 unit of Dynazyme™ (Finnzymes) or 2.6 units of Expand™ High Fidelity PCR System enzyme mix (Boehringer Mannheim Gmbh) using specific primers for each mRNA under study (see I–IV) The amplification included cycles of denaturation, annealing and extension under the conditions required for the primers and templates. In semi-quantitative PCR (II) serial dilutions of cDNA were amplified, and products from linear reaction rate were quantified with an image processing and analysing program (ScionImage PC, Scion Corporation).

4.4.2 Quantitative analysis of mRNA (I)

For quantitation of the mRNA amount of both proα1(I) collagen and β-actin in samples, cRNA controls for proα1(I) collagen and β-actin were used (Tasanen *et al.* 1996). These cRNA molecules contained small deletions, and served as the internal controls for a reverse transcription and for amplification efficiency. Due to the size difference between cRNA and endogenous mRNA, PCR products were easily separated by gel electrophoresis. In the method 0.1–2 µg of total cellular RNA and 10^8–10^9 molecules of cRNA were reverse transcribed in combination into cDNA. Serial dilutions of the cDNA mixture were amplified by specific primers, of which the upstream primer was ^{32}P end-labeled. Products were resolved by gel electrophoresis and autoradiographed. The amounts of radioactivity in the corresponding bands were determined by liquid scintillation, and plotted against the cRNA and RNA concentrations of samples. The copy number of target mRNA was obtained by extrapolating against the standard curve drawn with the Excel program (Microsoft) as described before (Tasanen *et al.* 1996).

4.4.3 Real-Time PCR (IV, Figures 5–14)

Specific TaqMan® primers and probes were designed with the Primer Express 1.0 software (Applied Biosystems). Sequences for the human MMP probes and primers are under the copyright of Applied Biosystems. The internal fluorogenic probes were labeled at the 5' end with the reporter dye FAM, at the 3' end with the quencher dye TAMRA and phosphate-blocked at the 3' end to prevent extension. The 18S rRNA probe was labeled with the VIC reporter dye at its 5' end and the TAMRA quencher dye at its 3' end. The amplified PCR products were quantified by measuring the accumulation of fluorescence during the amplification in each PCR cycle with the ABI Prism 7700 sequence detection system (Applied Biosystems). A cycle threshold (C_T) describes a cycle when the reporter fluorescence dye of a given sample becomes significantly different from the baseline signal. The C_T values obtained were plotted against log input RNA concentration in samples in serially diluted total RNA, and used to generate standard curves for all mRNAs analyzed. The amount of specific mRNA in samples were calculated from the standard curve, and normalized with the 18S rRNA.

4.4.4 Ribonuclease protection assay (RPA) (III)

The method of RPA III™ Ribonuclease Protection Assay (Ambion Inc.) was used for the study of the expression of MMP-14 mRNA in growth factor stimulated odontoblasts and pulp tissue. 3 to 5 µg of total RNA was hybridized with [α-^{32}P]-UTP- labeled MT1-MMP antisense RNA probe (nucleotides 218–638), treated with RNase A/Rnase T1 mixture and electrophoresed by a 5% denaturing PAGE gel. The gel was exposed to the X-ray film and bands were analyzed with an image processing and analysing program (ScionImage PC, Scion Corporation).

4.4.5 Verifying amplification products (II, III)

Either Southern blotting procedure or sequencing was used for validating identities of amplification products as follows.

4.4.5.1 Southern blot (II)

The specific MMP-8 cDNA probe was prepared by amplifying cDNA, synthesized from total RNA of gingival fibroblasts, with MMP-8 specific primers (Hanemaaijer *et al.* 1997). The product (522 bp) was purified with Qiaex Gel Extraction -method (QIAGEN) and labeled with [α-^{32}P]-dCTP. PCR products were fractioned on 1.5% agarose gel, transferred to nylon filter (Amersham) and hybridized to the MMP-8 probe.

4.4.5.2 Sequencing (II, IV)

PCR products were sequenced with sense or antisense primer using DNA Sequencing Kit according the manufacturer's instructions (Applied Biosystems). The data was analyzed with BLAST search tool provided by NCBI.

4.5 Analysis of protein synthesis and secretion

4.5.1 Western blot (II–IV, Figures 5–7, 14)

Samples were resolved by SDS-PAGE and transferred onto a nitrocellulose filter (Schleicher & Schuell or Hoefer Scientific Instruments) or PVDF filters (Immobilon™-P, Millipore Corporation). For the study of the protein secretion of MMP-1, MMP-10, MMP-13, TIMP-1, TIMP-2 and TIMP-3, conditioned culture media from odontoblasts and pulp tissue were collected and pooled within groups (for control n = 20, TGF-β1 n = 20, BMP-2 n = 15 and TGF-β1+BMP-2 n = 15). 3 µl of the pooled media in 1 x reduced Laemmli buffer were subjected to ECL Western blot. The filters were reacted with either a monoclonal or a polyclonal antibody specific for proteins described in studies II to IV. For this thesis the following antibodies were also used: polyclonal MMP-1 antibody (0.2 µg/ml) (Chemicon International, Inc.), a polyclonal MMP-10 antibody (1 µg/ml) (Oncogene™), a polyclonal MMP-13 antibody (0.3 µg/ml), (Chemicon International, Inc.), a monoclonal TIMP-1 antibody (1:700) (Triple Point Biologies), a monoclonal TIMP-2 antibody (1µg/ml) (Immunodiagnostic Oy), and a monoclonal TIMP-3 antibody (5 µg/ml) (Oncogene™). Immunoreactive bands were visualized either by 3,3-diaminobenzidine tetrahydrochloride (DAB) (II) or by ECL Chemiluminescence Western blot method (Amersham Pharmacia Biotech) according to supplier's procedures (II–IV, Figs 1–3, 10).

4.5.2 Enzymography (IV)

The presence of secreted gelatinases was assayed with the use of an enzymography in 0,75 mm thick 10% SDS-PAGE gels impregnated with 1 mg/ml gelatin, which had been labeled fluorescently with 2-methoxy-2,4-diphenyl-3-(^{2}H)furanone (Fluka). Samples were electrophoresed without reduction, and then gel was incubated in 50 mM Tris-HCl buffer, pH 7.8, containing 150 mM NaCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, at 37°C. The degradation of gelatin was visualized under long wave UV light, and finally the gel was stained with Coomassie Brilliant Blue.

4.5.3 Immunofluorescence assay (II)

The amount of MMP-8 secreted into the culture medium was determined by an immunofluorometric assay as described (Hanemaaijer *et al.* 1997). The conditioned culture medium was diluted in assay buffer and incubated with MMP-8 tracer antibody (Hanemaaijer *et al.* 1997). Fluorescence was measured after the addition of enhancement solution with a 1234 Delfia Research Fluorometer (Wallac).

4.5.4 Immunohistochemical staining (II)

Frozen sections of intact teeth were stained by Vectastain Elite kit as recommended by supplier (Vector Laboratorios). The sections were incubated with the specific MMP-8 polyclonal antibody (Michaelis *et al.* 1990). In the control sections, normal rabbit serum was used instead of the primary antibody. Then the sections were incubated with biotinylated anti-rabbit IgG, the avidin-biotin-peroxidase complex, and 3-amino-9-ethylcarbazole (Sigma) or DAB with a DAB enhancing solution was used for the detection (both from Vector Laboratories). Finally, the sections were counterstained with haematoxylin.

4.5.5 Immunoassays for type I and III collagen domains (I)

Radioimmunoassays for the aminoterminal propeptide of type I procollagen (PINP) (Melkko *et al.* 1996) and the aminoterminal propeptide of type III collagen (PIIINP) (Risteli *et al.* 1988) were used to measure their concentrations in the conditioned culture media as detailed described in study I.

4.6 ProMMP activation assay (III)

To analyze the conversion of proMMP-20 and proMMP-2 into active forms by MT1-MMP, soluble pro- and catalytic domains of MT1-MMP and soluble proMT1-MMP (Invitek GmbH) were activated with TAT-2 (Sorsa *et al.* 1997). These activated MT1-MMPs constructs were used in the activation reaction with odontoblasts, conditioned odontoblast culture media and pro-recombinant human MMP-20. In addition to MT1-MMPs, activation by APMA was also studied. Results were analyzed by ECL-Western blotting or Coomassie protein staining procedure. In the case of MMP-2, zymography was used. Activation was followed by the conversion on enzyme molecular weight.

4.7 Statistical analysis (III, IV, Figures 5–14)

One-way analysis of variance, ANOVA, was used to determine the statistical significance of the differences in the mRNA expression levels in odontoblasts and pulp tissue, between different growth factor treatments observed with either RPA or Real-Time PCR procedures, with the SPSS 10 program (SPSS Inc.). When ANOVA detected statistically significant differences, LSD *P*-values were used to describe the significance of the differences between the individual groups.

5 Results

5.1 Expression of proα1(I) collagen in mature human odontoblasts and pulp tissue, and in pulpal and gingival fibroblasts and bone osteoblasts (I)

Quantities of proα1(I) collagen mRNA molecules between odontoblasts, pulp tissue and pulpal fibroblasts varied. Odontoblasts expressed 1278 proα1(I) molecules/pg total RNA, which was approximately 6-fold more compared to amounts of proα1(I) collagens in pulp tissue and pulpal fibroblasts (214 and 234 molecules/pg total RNA, respectively). The highest amount of proα1(I) collagen mRNA was expressed in both osteoblasts (8058 molecules of proα1(I) collagen/pg total RNA) and gingival fibroblasts (6073 proα1(I) collagen/pg total RNA).

5.2 Regulation of type I and III collagens by TGF-β1 in mature human odontoblasts and pulp tissue, and in pulpal and gingival fibroblasts (I)

TGF-β1 had no clear effect on proα1(I) collagen mRNA levels or type I procollagen protein synthesis either in odontoblasts or pulp tissue. Odontoblasts also secreted type III procollagen during the culture period, with no clear effect of TGF-β1 on its synthesis level. TGF-β1 up-regulated proα1(I) collagen mRNA expression up to 3-fold in gingival fibroblasts. However, the effect of TGF-β1 on the proα1(I) collagen mRNA in pulpal fibroblasts depended on culture conditions: when serum was included in the culture medium, no response to TGF-β1 was observed, but without serum, the response was over 6-fold.

5.3 Expression of MMPs and TIMPs, and regulation by TGF-β1 and BMP-2 in mature human odontoblasts and pulp tissue (II–IV, Figures 5–14)

An overview of all results is given after the detailed description of each separate MMP (see Table 2). Even though the expression of almost all MMPs was analyzed with Real-Time PCR, the method does not allow comparison of the expression levels between individual genes. Only changes in expression level within individual gene expression could be estimated.

5.3.1 Simple hemopexin-domain containing MMPs

5.3.1.1 Collagenases (II, IV, Figures 5, 6)

MMP-8 mRNA was expressed by mature human odontoblasts and pulp tissue in both native and cultured tissues, and by pulpal fibroblasts (II). Bone marrow cells, known to express MMP-8, served a positive control showing the expression of MMP-8 mRNA. By Western blot analysis, a 65 kDa immunoreactive MMP-8 form was detected in native odontoblasts and pulp tissue, and also a 50 kDa form in native pulp tissue. Both tissues also secreted 50 kDa MMP-8 into conditioned culture media, and no 75 to 80 kDa PMN-MMP-8 forms were observed in either sample. Immunohistochemical staining confirmed the production of MMP-8 in odontoblasts. In addition, some endothelial cells, located in the pulp tissue vessels, expressed MMP-8. TGF-β1 clearly decreased MMP-8 mRNA and protein levels in both odontoblasts and pulp tissue. (II)

Real-Time PCR showed that MMP-1 mRNA was expressed by both native odontoblasts and pulp tissue (IV). The expression level of MMP-1 seems to be very low in native samples, since conventional RT-PCR did not detect MMP-1 either in native odontoblasts or pulp tissue. However, cultured odontoblasts and pulp tissue expressed MMP-1 mRNA (IV). In addition, TGF-β1 and BMP-2 affected MMP-1 gene expression in both odontoblasts (ANOVA, $F = 4.643$, $P = 0.043$) and pulp tissue (ANOVA, $F = 3.460$, $P = 0.048$) (Fig. 5A). A trend could be seen that TGF-β1 and BMP-2 inhibited the expression of MMP-1 mRNA in odontoblasts, and that TGF-β1 and BMP-2 in combination modestly increased the MMP-1 mRNA level compared to control (Fig. 5A), although LSD analysis did not show statistical significance between growth factor treatments compared to control. In pulp tissue, BMP-2 significantly increased the expression of MMP-1 mRNA by 2.8-fold compared to control ($P = 0.009$), and a similar trend could be seen with TGF-β1, which, however, did not reach statistical significance ($P = 0.090$) (Fig. 5A). By Western blotting, immunoreactive bands corresponding most likely to latent 52 kDa MMP-1 and active 42 kDa MMP-1 were detected in the conditioned culture media of both tissues (Fig. 5B).

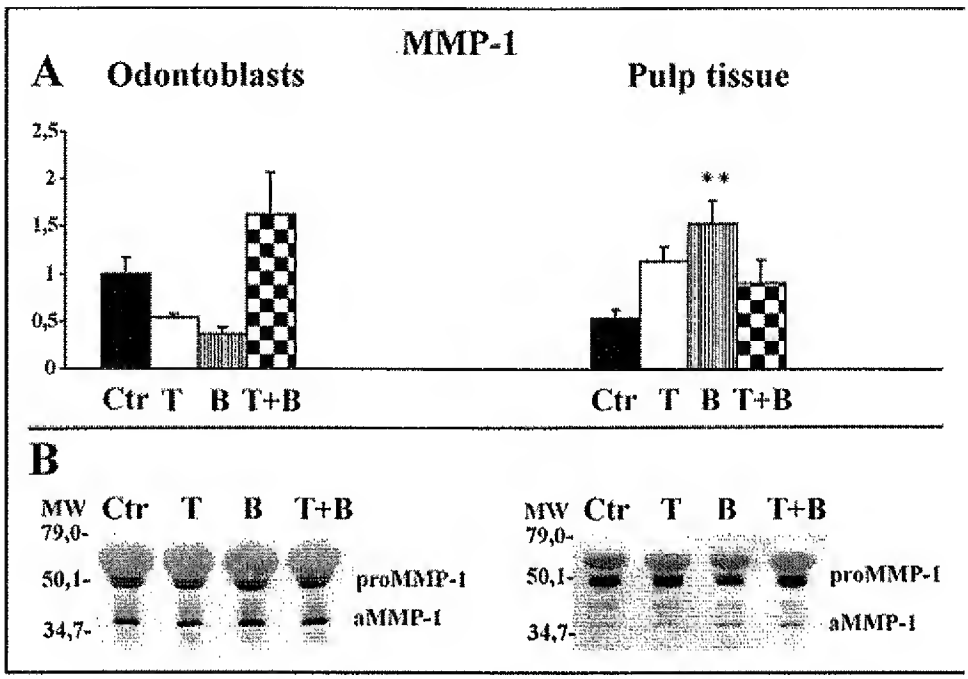

Fig. 5. A) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-1 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-1 mRNA expression in odontoblasts (n=2–3 in all groups) and pulp tissue (n=3–5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$). B) MMP-1 protein secretion into conditioned culture media of odontoblasts and pulp tissue was analyzed by Western blot procedure, which show the latent (proMMP-1) and active MMP-1 (aMMP-1) immunoreactive bands in all samples.

MMP-13 mRNA was expressed 5-fold more in native pulp tissue compared to that in native odontoblasts (IV). However, the level of MMP-13 mRNA in cultured odontoblasts was below the detection level assayed with Real-Time PCR procedure. Instead, cultured pulp tissue expressed MMP-13 mRNA, and BMP-2 significantly increased the expression of MMP-13 by 4.6-fold compared to control ($P = 0.004$), with no marked effect observed with TGF-β1, either alone or in combination with BMP-2 (Fig. 6A). The active form of approximately 50 kDa of MMP-13, with no latent 60 kDa MMP-13, was detected in the conditioned culture media of pulp tissue samples (Fig. 6B).

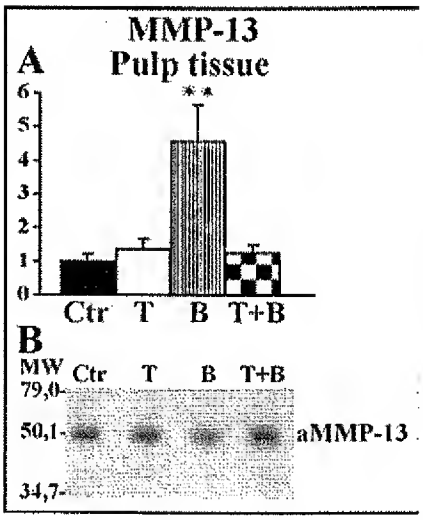

Fig. 6. A) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-13 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-13 mRNA expression in pulp tissue (n=4–5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$). B) MMP-13 protein secretion into conditioned culture media of pulp tissue was analyzed by Western blot procedure, which shows the active MMP-13 (aMMP-13) immunoreactive bands in all samples.

5.3.1.2 Stromelysins (IV, Figure 7)

MMP-3 expression was below detection in both native odontoblasts and pulp tissue assayed with Real-Time-PCR. The expression of MMP-3 in cultured odontoblasts and pulp tissue was controversial, and remains to be further evidenced (IV). Another stromelysin, MMP-10, was expressed 27-fold more by native odontoblasts compared to that in native pulp tissue (IV). However, in the cultured pulp tissue the level of MMP-10 in the control (Ctr) samples was 4-fold higher compared to amount of that in cultured odontoblasts (Fig. 7A, B). While no statistically significant differences were detected in the MMP-10 mRNA level between different growth factor groups in odontoblasts (ANOVA, $F = 1.501$, $P = 0.279$) (Fig. 7A), BMP-2 significantly up-regulated MMP-10 mRNA by 1.9-fold in pulp tissue ($P = 0.023$) (Fig. 7B). By Western blotting faint immunoreactive forms of both latent 54 kDa and active 44 kDa MMP-10 were detected in the conditioned culture media of odontoblasts (Fig. 7C). In the conditioned culture media of pulp tissue immunoreactive forms of both latent and active MMP-10 were also observed, however they were quite faint (not shown).

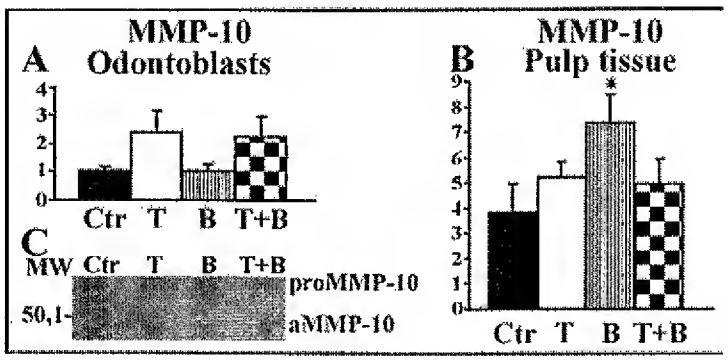

Fig. 7. A, B) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-10 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-10 mRNA expression in odontoblasts (A) (n=3–4 in all groups) and pulp tissue (B) (n=4–5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$). C) MMP-10 protein secretion into conditioned culture media of odontoblasts was analyzed by Western blot procedure, which shows the latent (proMMP-10) and active MMP-10 (aMMP-10) immunoreactive bands in all samples.

5.3.1.3 Others (IV, Figure 8)

MMP-20 was expressed 67-fold more in native odontoblasts compared to the amount in native pulp tissue (IV). MMP-20 mRNA expression was not regulated by either TGF-β1 or BMP-2 in either cultured odontoblasts or pulp tissue (ANOVA, $F = 2.253$, $P = 0.135$; $F = 0.072$, $P = 0.974$, respectively) (IV). With the use of Western blotting, both latent 51 kDa and active 48 kDa MMP-20 immunoreactive bands were detected in the conditioned culture media of odontoblasts (IV). Additionally, a faint 29 kDa species of MMP-20 was observed only in odontoblast media. In conditioned culture media of pulp tissue, MMP-20 was detected mostly as the latent form of 51 kDa (IV).

MMP-12 mRNA was expressed by neither odontoblasts or pulp tissue, nor in native or cultured samples (IV). Instead, MMP-19 mRNA was equally expressed by both native and cultured odontoblasts and pulp tissue (IV, Fig. 8A), whereas MMP-27 was expressed approximately 10-fold more in the control (Ctr) sample of the cultured odontoblasts compared to the respective control (Ctr) of the pulp tissue (Fig. 8B). MMP-27 mRNA in native odontoblasts and pulp tissue was not analyzed. Growth factors did not have statistically significant effects on either MMP-19 (Fig. 8A) or MMP-27 (Fig. 8B) mRNA expression in odontoblasts (ANOVA, $F = 1.775$, $P = 0.225$; $F = 0.948$, $P = 0.448$, respectively) and pulp tissue (ANOVA, $F = 0.981$, $P = 0.426$; $F = 0.296$, $P = 0.828$, respectively).

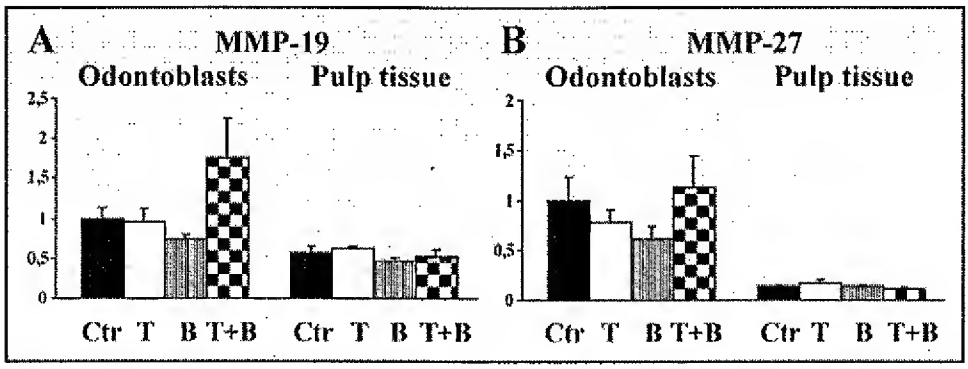

Fig. 8. A, B) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-19 mRNA and MMP-27 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-19 mRNA (A) and MMP-27 mRNA (B) expression in odontoblasts (n=3–5 in all groups) and pulp tissue (n=4–5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$).

5.3.2 Gelatin-binding MMPs (IV, Figure 9)

MMP-9 mRNA was equally expressed by both odontoblasts and pulp tissue in both native and cultured tissues (IV). TGF-β1, alone or in combination with BMP-2, significantly up-regulated MMP-9 mRNA expression by 2.4- and 2.6-fold, respectively, compared to control (P = 0.018 and p = 0.014, respectively), while BMP-2 had no effect in odontoblasts. These growth factors did not markedly affect MMP-9 mRNA expression in pulp tissue (ANOVA, F = 0.245, P = 0.864). Enzymographic analysis detected gelatinolytic activities corresponding to proMMP-9 in conditioned culture media of both tissues, while gelatinolytic activity at size 78 kDa, corresponding to active MMP-9, could be observed only in the odontoblast conditioned culture media. (IV)

MMP-2 mRNA was expressed 5-fold more in native odontoblasts compared to the amount in native pulp tissue, while in cultured tissues the MMP-2 mRNA level was quite similar (IV, Fig. 9). In odontoblasts, TGF-β1 and BMP-2 in combination induced the expression of MMP-2 mRNA significantly by 1.9-fold compared to control (P = 0.001) (Fig. 9). In pulp tissue, both TGF-β1 and BMP-2 alone significantly increased the MMP-2 mRNA level by 1.8-fold and 2.1-fold, respectively, compared to control (P = 0.019 and P = 0.002, respectively) (Fig. 9). MMP-2 gelatinolytical activities (65 kDa and 57 kDa for pro- and active forms of MMP-2, respectively) were observed in odontoblast conditioned culture media, while in pulp tissue conditioned culture media only the proform could be detected (IV).

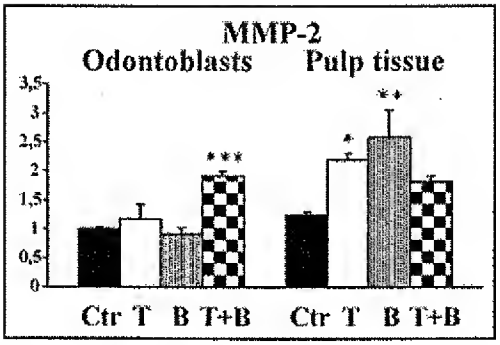

Fig. 9. The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-2 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-2 mRNA expression in odontoblasts (n=3–4 in all groups) and pulp tissue (n=4 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$).

5.3.3 Furin-activated secreted MMPs (IV)

MMP-11 mRNA was expressed 5-fold more abundantly in native odontoblasts compared to native pulp tissue (IV). However, the expression of MMP-11 mRNA was below detection level in both cultured odontoblasts and pulp tissue assayed with Real-Time PCR (not shown).

5.3.4 Minimal-domain MMPs (IV, Figure 10)

MMP-7 mRNA was expressed only by native and cultured odontoblasts, and TGF-β1 and BMP-2 had no clear effect on its expression level (ANOVA, $F = 1.630$, $P = 0.239$) (IV, Fig. 10A). MMP-26 mRNA was expressed approximately 5-fold more by cultured odontoblasts compared to cultured pulp tissue, with no clear effect of either TGF-β1 or BMP-2 on its regulation in odontoblasts and pulp tissue (ANOVA, $F = 1.902$, $P = 0.188$; ANOVA, $F = 0.296$, $P = 0.828$, respectively) (Fig. 10B). MMP-26 mRNA expression in native odontoblasts and pulp tissue was not analyzed.

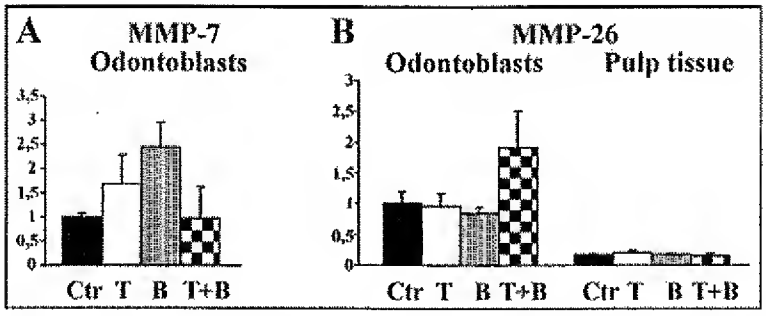

Fig. 10. A, B) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-7 mRNA and MMP-26 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-7 mRNA expression in odontoblasts (n=3–4 in all groups) (A) and MMP-26 mRNA expression in odontoblasts (n=3–4 in all groups) and pulp tissue (B) (n=5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$).

5.3.5 Type I transmembrane MMPs (III, IV, Figure 11)

MMP-14 mRNA was expressed by odontoblasts and pulp tissue, both in native and cultured tissues (III). The expression of MMP-14 mRNA was over 10 times more abundant in native odontoblasts compared to native pulp tissue (IV). Immunoreactive forms of 65 kDa and 51 kDa, corresponding to the latent and active forms of MMP-14, were detected in both cells. In addition, truncated forms were present (III). In odontoblasts, BMP-2 significantly down-regulated MMP-14 expression by 66% compared to control ($P = 0.006$), whereas TGF-β1 alone, or in combination with BMP-2, only slightly reduced the messenger RNA. In pulp tissue, similar effects were seen, but the reduction was not statistically significant (ANOVA, $P = 0.231$) (III).

In addition to MMP-14, other type I transmembrane MMPs were expressed by odontoblasts and pulp tissue, including MMP-15 and MMP-16 (IV, Fig. 11A, B). While the expression of MMP-15 and MMP-16 appears to be 3 to 4 times more abundant in native odontoblasts compared to native pulp tissue (IV), the levels of both mRNAs were quite similar in cultured tissues (Fig. 11A, B). MMP-24 mRNA expression was detected in native odontoblasts, but not in native pulp tissue (IV). However, the level of MMP-24 mRNA expression in both cultured tissues was below detection level (not shown). In odontoblasts, the combination of TGF-β1 and BMP-2 significantly up-regulated the MMP-16 mRNA expression by 3.8-fold compared to control ($P = 0.012$) (Fig. 11B), and no clear effect was seen with either growth factor on the mRNA expression of MMP-15 (ANOVA, $F = 2.838$, $P = 0.079$) (Fig. 11A). In pulp tissue, no regulatory effect on either MMP-15 or MMP-16 mRNA expressions could be observed (ANOVA, $F = 0.349$, $P = 0.790$; $F = 1.063$, $P = 0.392$, respectively) (Fig. 11A, B).

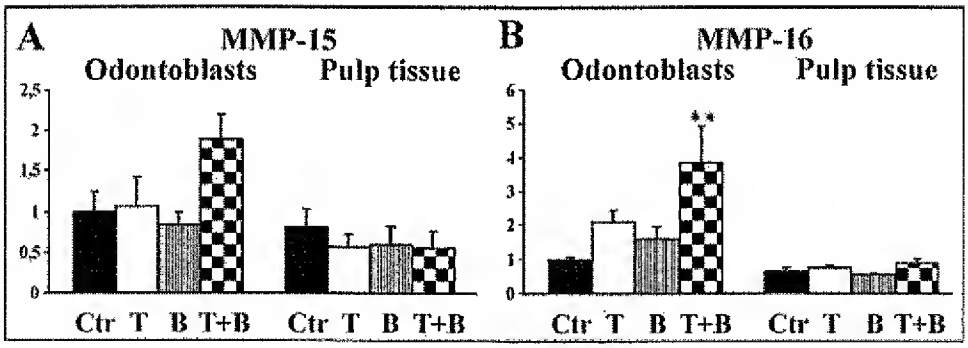

Fig. 11. A, B) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-15 mRNA and MMP-16 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-15 mRNA (A) and MMP-16 mRNA (B) expression in odontoblasts (n=3–5 in all groups) and pulp tissue (n=4–5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$).

5.3.6 GPI-linked MMPs (IV, Figure 12)

MMP-17 mRNA was expressed 3-fold more in native pulp tissue compared to native odontoblasts (IV). The expression of MMP-17 mRNA in cultured odontoblasts was below detection level (not shown). MMP-25 mRNA was expressed by native and cultured odontoblasts, and cultured pulp tissue (IV, Fig. 12B). A slight trend was seen with TGF-β1 and BMP-2, that either alone or in combination, they may induce the pulpal MMP-17 mRNA level, although findings were not statistically significant (ANOVA, $F = 1.960$, $P = 0.179$) (Fig. 12A). Growth factors did not significantly affect the mRNA expression level of MMP-25 either in odontoblasts (ANOVA, $F = 0.447$, $P = 0.726$) or in pulp tissue (ANOVA, $F = 0.434$, $P = 0.732$) (Fig. 12B).

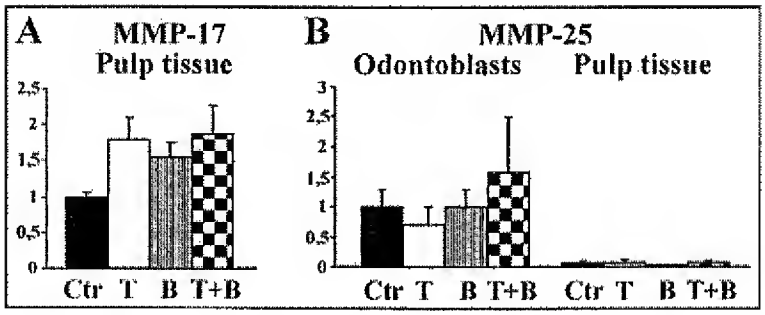

Fig. 12. A, B) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-17 mRNA and MMP-25 mRNA expression were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative MMP-17 mRNA expression in pulp tissue (A) (n=3–4 in all groups) and MMP-25 mRNA expression in odontoblasts (n=3 in all groups) and pulp tissue (B) (n=5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$).

5.3.7 Type II transmebrane MMPs (IV, Figure 13)

MMP-23 was expressed approximately 5-fold more by odontoblasts and pulp tissue in both native and cultured tissues, and only treatment of odontoblasts with TGF-β1 and BMP-2 in combination increased the mRNA level of MMP-23 by 1.4-fold compared to control ($P = 0.056$) (IV, Fig. 13). In pulp tissue, no regulatory effect of growth factors on MMP-23 mRNA expression was observed (ANOVA; $F = 102$, $P = 0.957$) (Fig. 13).

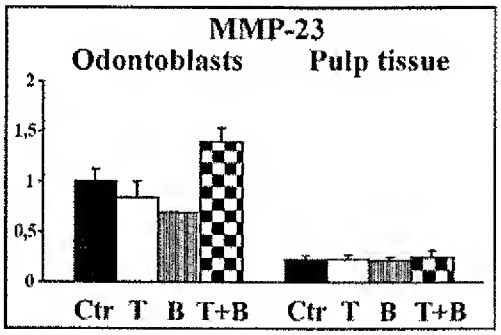

Fig. 13. The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on MMP-23 mRNA expression were analyzed with Real-Time PCR. Graphic presentation shows mean and standard error of the mean (SEM) of the relative MMP-23 mRNA expression in odontoblasts (n=3–4 in all groups) and pulp tissue (n=3–4 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$).

5.3.8 TIMPs (IV, Figure 14)

TIMP-1, -2 and -3 were expressed by odontoblasts and pulp tissue, in both native and cultured tissues (IV, Fig. 14 A–F). TIMP-4 mRNA expression was analyzed only in cultured tissues (Fig. 14G). While the expression of TIMP-2 mRNA was similar in both cultured odontoblasts and pulp tissue (Fig. 14C), TIMP-1, TIMP-3 and TIMP-4 were expressed slightly more abundantly in cultured pulp tissue compared to cultured odontoblasts (Fig. 14A, E, G). In odontoblasts, TGF-β1 in combination with BMP-2 increased TIMP-1 mRNA expression by 2.1-fold ($P = 0.046$) (Fig. 14A) and TIMP-3 mRNA by 2.0-fold ($P = 0.043$) (Fig. 14E), whereas TIMP-2 mRNA (Fig. 14C) and TIMP-4 mRNA (Fig. 14G) were expressed constitutively (ANOVA, $F = 1.057$, $P = 0.406$; ANOVA, $F = 0.229$, $P = 0.872$, respectively). In pulp tissue, both TGF-β1 alone, and in combination with BMP-2, up-regulated TIMP-3 mRNA expression by 1.5-fold and 1.7-fold, respectively ($P = 0.056$; $P = 0.013$, respectively) (Fig. 14E). On the contrary, TGF-β1, BMP-2 and TGF-β1 in combination with BMP-2 down-regulated the TIMP-4 mRNA by 45%, 35% and 47% respectively ($P = 0.002$; $P = 0.012$; $P = 0.001$, respectively) (Fig. 14G). By Western blotting, immunoreactive bands of 28.5 kDa TIMP-1 (Fig. 14B), 21 kDa TIMP-2 (Fig. 14D) and 27 kDa TIMP-3 (Fig. 14F) were detected in both tissues. TIMP-4 protein secretion was not analyzed.

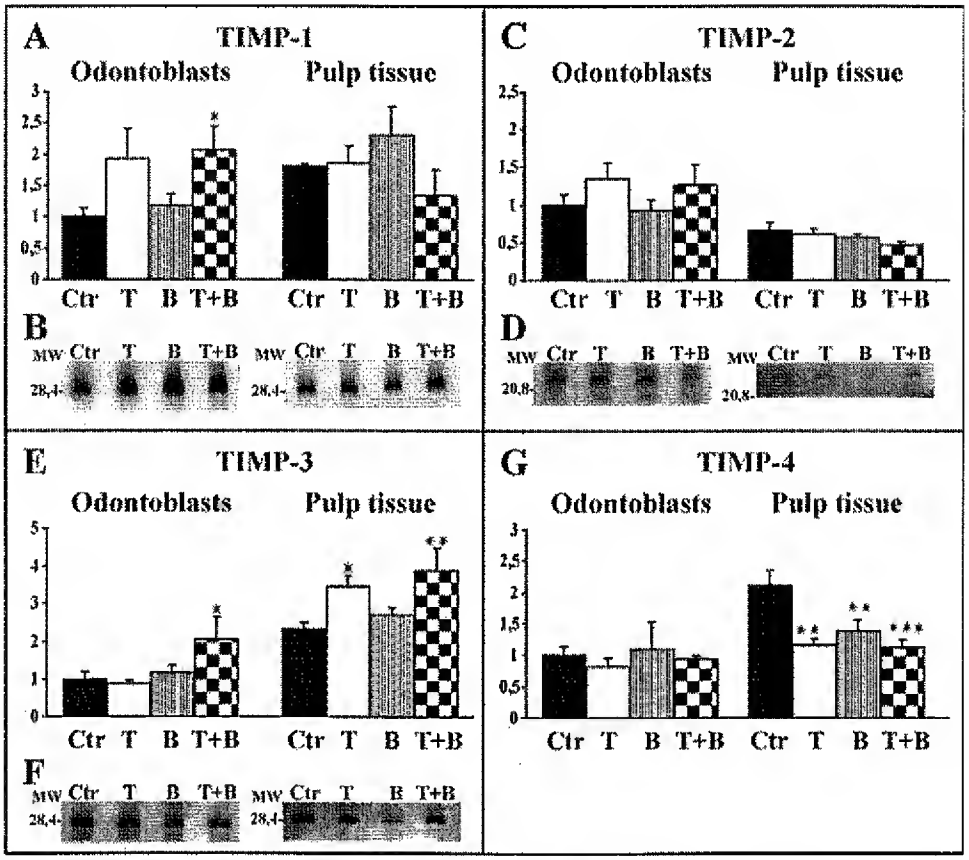

Fig. 14. A, C, E, G) The effects of 1 ng/ml TGF-β1 and 100 ng/ml BMP-2 on TIMP-1 (A), TIMP-2 (C), TIMP-3 (E) and TIMP-4 (G) mRNA expression levels were analyzed with Real-Time PCR. Graphic presentations show mean and standard error of the mean (SEM) of the relative TIMPs expression levels in odontoblasts (n=3–5 in all groups) and pulp tissue (n=3–5 in all groups) after tissue stimulation with either TGF-β1 (T), BMP-2 (B), TGF-β1 combined with BMP-2 (T+B) or without mediator (Ctr). One-way ANOVA with LSD test was used for statistical analysis (*: $P = 0.05$, **: $P = 0.01$, ***: $P = 0.001$). B, D, F) TIMP protein secretion into conditioned culture media of odontoblasts and pulp tissue was analyzed by Western blot, which show the immunoreactive bands of the respective size of 28.5 kDa TIMP-1 (B), 21 kDa TIMP-2 (D) and 27 kDa TIMP-3 (F) in all samples.

Table 2. Overview of the expression levels of MMPs and TIMPs in mature human odontoblasts and pulp tissue, and the effect of growth factors (GFs), TGF-β1 (T) and BMP-2 (B), on their expression.

MMP/TIMP	Native cells		Cultured cells		Effect of GFs		
					T	B	T+B
	OBs	Pulp	OBs	Pulp	OB/Pulp	OB/Pulp	OB/Pulp
Simple hemopexin-domain containing MMPs							
MMP-1	+/–	+/–	+	+	↓/↑	↓/↑↑↑	↑/–
MMP-8	+	+	+	+	↓↓↓/↓↓↓	NS	NS
MMP-13	+	++	ND	++	ND/–	ND/↑↑↑	ND/–
MMP-3	ND	ND	+/–	+/–	ND	ND	ND
MMP-10	++	+/–	+	+	↑/–	–/↑↑↑	↑/–
MMP-12	ND	ND	ND	ND	ND	ND	ND
MMP-19	+	+	+	+	–/–	–/–	↑/–
MMP-20	+++	+	+++	+	–/–	–/–	–/–
MMP-27	NS	NS	+	+/–	–/–	–/–	–/–
Gelatin-binding MMPs							
MMP-2	+++	++	++	++	–/↑↑↑	–/↑↑↑	↑↑↑/↑
MMP-9	+	+	+	+	↑↑↑/–	–/–	↑↑↑/–
Furin-activated secreted MMPs							
MMP-11	+	+/–	ND	ND	ND	ND	ND
Minimal-domain MMPs							
MMP-7	+	ND	+	ND	↑/ND	↑↑/ND	–/ND
MMP-26	NS	NS	+	+/–	–/–	–/–	↑/–
Type I transmembrane MMPs							
MMP-14	+++	++	+++	+++	↓/↓	↓↓↓/↓↓	↓/↓
MMP-15	+	+	+	+	–/–	–/–	↑/–
MMP-16	+	+	+	+	–/–	–/–	↑↑↑/–
MMP-24	+/–	ND	ND	ND	ND	ND	ND
GPI-linked MMPs							
MMP-17	+	+++	ND	+++	ND/↑	ND/↑	ND/↑
MMP-25	+	ND	+	+/–	–/–	–/–	–/–
Type II transmembrane MMPs							
MMP-23	+	+/–	+	+/–	–/–	–/–	↑↑↑/–
TIMPs							
TIMP-1	++	++	++	++	↑/–	–/–	↑↑↑/–
TIMP-2	++	++	++	++	–/–	–/–	–/–
TIMP-3	+	++	+	++	–/↑↑↑	–/–	↑↑↑/↑↑↑
TIMP-4	NS	NS	+	++	–/↓↓↓	–/↓↓↓	–/↓↓↓

+++: strong expression; ++: intermediate expression; +: low expression; +/–: very low expression; ND: no expression detected; ↑↑↑: significant up-regulation; ↑↑: intermediate up-regulation; ↑: low or no up-regulation, –: no effect; ↓↓↓: significant down-regulation; ↓↓: intermediate down-regulation; ↓: low or no down-regulation
NS: not studied

5.4 Activation of proMMP-20 and proMMP-2 by MT1-MMP (III)

Active MT1-MMP was able to convert proMMP-20 into the active form of MMP-20 in cultured odontoblasts, while this effect was less pronounced in conditioned odontoblast cultured media. In addition, recombinant human proMMP-20 was converted into the active form by MT1-MMP. MT1-MMP also activated proMMP-2, especially in odontoblasts. APMA treatment of odontoblasts and odontoblast conditioned culture media induced both proMMP-20 and proMMP-2 activation.

6 Discussion

6.1 The expression of and effects of TGF-β1 on type I and type III collagens in human dentin-pulp complex (I)

Type I collagen is a fundamental component of a fibrous organic framework of dentin. However, there is evidence that the dentin organic matrix also contains other collagens. Collagens are triple helix molecules of either homo- or heteropolymers, but only proα2(I) (Lukinmaa *et al.* 1992) and proα1(III) (Lukinmaa *et al.* 1993) collagen mRNA have been shown to be expressed by human odontoblasts. Even though some animal studies suggest that type III collagen is not synthesized by odontoblasts (Munksgaard *et al.* 1978, 1980, d'Souza *et al.* 1995), others have detected type III collagen (Karjalainen *et al.* 1986) or procollagen (Becker *et al.* 1986) in human predentin and odontoblasts, in the dentin of dentinogenesis imperfecta-patients (Karjalainen & Söderling 1984, Waltimo *et al.* 1994) and in human reparative dentin (Magloire *et al.* 1988b). Also type VI collagen has been observed in intact dentin (Becker *et al.* 1986).

Even though odontoblast secretion has been investigated for years, surprisingly little is known about the expression and regulation of the collagens in human odontoblasts and pulp tissue, since most of the studies have been performed with animal models. The development of an organ culture method for mature human dentin-pulp complex tissues has enabled investigation of the secretory activity of both mature human odontoblasts and pulp tissue (Tjäderhane *et al.* 1998b). With this method, mRNA of proα1(I) chain of type I collagen, and type I procollagen were found to be expressed by odontoblasts and pulp tissue, and that odontoblasts abundantly synthesized type I collagen protein compared to the respective amount of pulp tissue (I), confirming previous findings (Lukinmaa *et al.* 1992, 1993). Furthermore, this work showed for the first time that mature human odontoblasts indeed synthesize and secrete type III collagen protein (I). The expression of type III collagen in pulp tissue was not analyzed, since it has been well characterized that pulp tissue synthesizes type III collagen (van Amerongen *et al.* 1983).

One reason for the apparent discrepancy between these results and some early animal experiments (Munksgaard *et al.* 1978, 1980) may arise from differences in protein composition between different species (Linde & Goldberg 1993). Alternatively, in the

earlier studies, the extensive purification processes might have caused the loss of minor collagenous components (Karjalainen *et al.* 1986). For example, pepsin treatment has been shown to lead to marked underestimation of type III collagen levels in other tissues (Burke *et al.* 1977). The reason for type III collagen expression by mature intact human odontoblasts is unclear, but it may be needed for organization of the dentin organic matrix prior to mineralization, since type III collagen is a regular component of soft tissue ECM, but is not present in normal mineralized human tissues, including bone and dentin (*e.g.* Becker *et al.* 1986). The virtual absence of type III collagen in physiological human dentin suggests that the protein is degraded and/or removed from the predentin during the organization of the matrix. In pathological situations, this degradation and removal may be hindered and type III collagen may be left in the predentin and mineralized dentin, possibly because of alterations in MMP expression. Type III collagen may, in turn, influence the structure of dental tissues (Lukinmaa *et al.* 1993).

TGF-β1 has been suggested to increase protein synthesis, including type I collagen, as a response to dental injury (Smith *et al.* 1995, Sloan & Smith 1999), but the precise mechanisms of how TGF-β1 regulates the responses to injury are still not well known. For example, Sloan and Smith (1999) suggest that the increase in the predentin width after TGF-β treatment is due to increased odontoblast matrix synthesis. However, TGF-β has been shown to reduce the expression of proteins involved in dentin matrix mineralization, such as dentin sialophosphoprotein and alkaline phosphatase (Ibbotson *et al.* 1989, Shibata *et al.* 1993, Nakashima 1992, Shirakawa *et al.* 1994). Therefore, the predentin widening observed in TGF-β1 treated rodent incisor slices may be caused by the reduced organic matrix mineralization. This assumption is supported by *in vitro* studies with the human odontoblast culture method showing that TGF-β may not induce dentin formation by up-regulating collagen synthesis, since TGF-β1 had no effect on either type I or type III collagen levels in mature human odontoblasts (I). These findings are further supported by a study from Shibata and colleagues (1993), showing that collagen synthesis levels in osteoblasts are affected by the state of cell differentiation, and in mature osteoblasts TGF-β1 does not affect collagen synthesis. Alternatively, these seemingly conflicting findings may again reflect the differences between species that have been demonstrated in several other cases (Linde & Goldberg 1993).

On the other hand, the data shows that TGF-β1 up-regulates proα1(I) collagen mRNA in other cell types such as gingival fibroblasts (I), which is in accordance with other studies (Wrana *et al.* 1986, James *et al.* 1998). Pulpal fibroblasts responded variably to a TGF-β stimulus, and the effect was highly dependent on culture conditions (I), as has been previously suggested (Moule *et al.* 1995, Stanislawski *et al.* 1997). In addition, the expression of proα1(I) collagen level in pulpal fibroblasts was different from that of pulp tissue after TGF-β treatment in serum free conditions (I).

Taken together, the present data suggests that TGF-β has no direct effect on the synthesis of collagens in mature human odontoblasts. However, TGF-β may still indirectly affect dentin matrix formation and mineralization by either regulating the differentiation of replacement odontoblasts or by regulating the expresson of other proteins such as matrix metalloproteinases. The conflicting results with the pulp fibroblast cultures under different culture conditions confirm the previous suggestion that the traditional cell cultures with the pulpal cells do not necessarily provide results that can be interpreted as the same as an *in vivo* situation (Moule *et al.* 1995, Stanislawski *et al.*

1997). Organ culture models, such as used here (I–IV), or the human tooth slice culture-technique (Melin *et al.* 2000), provide important tools to investigate the responses of different tissue types to various stimuli, and to compare results between different species.

6.2 Expression of MMPs and regulation by TGF-β1 and BMP-2 in human dentin-pulp complex (II–IV, Figures 5–14)

6.2.1 Collagenases (II, IV, Figure 5, 6)

Cells synthesizing mineralized tissue, such as osteoblasts and chondrocytes, produce collagenases (Johansson *et al.* 1997, Takiguchi *et al.* 1998, Shlopov *et al.* 1999, Wang *et al.* 2002), and unidentified collagenolytic activity has also been detected in human dentin (Dayan *et al.* 1983, Dumas *et al.* 1985). The present study shows that mature human odontoblasts and pulp tissue express MMP-1 and MMP-8, whereas MMP-13 was exclusively expressed by pulp tissue (II, IV, Figs 5, 6). The MMP-1 expression level in healthy dentin-pulp complex cells was very low, but increased in cultured cells for an unknown reason (IV). Whether the expression of collagenases is induced under pathological conditions remains to be shown, since studies with human osteoblasts and pulp fibroblasts have shown that collagenase expression can be stimulated with cytokines (Meikle *et al.* 1992, Tamura *et al.* 1996, Lin SK *et al.* 2001). By Western blot analysis, both latent and active immunoreactive forms of MMP-1 protein could be detected in the conditioned culture media of odontoblasts and pulp tissue. However, only the immunoreactive form corresponding to active MMP-13 was observed in pulp tissue conditioned culture media. The absence of latent MMP13 in conditioned culture media indicates that MMP-13 is activated at the cell membrane during secretion, as has been suggested by Knäuper and colleagues (1996c). This cell membrane associated activation, possibly by MMP-2–MT1-MMP in combination (Knäuper *et al.* 1996c) would explain the absence of the latent form in conditioned culture media. The protein size of MMP-8 depends on its glycosylation state, and 50 kDa MMP-8 has previously been detected as secreted by chondrocytes, the so called mesenchymal-type MMP-8 (Cole *et al.* 1996), which is in line with our observation (II).

Collagenases seem to be down-regulated by TGF-β in cells synthesizing calcified matrix, since mRNAs of both MMP-1 and MMP-8 were down-regulated by TGF-β1 in odontoblasts (II, Fig. 5) as they are in chondrocytes (Shlopov *et al.* 1999, Wang *et al.* 2002). This is consistent with the information that the TGF-β inhibitory element, TIE, in the MMP-1 promoter is responsible for inhibition of gene transcription (White *et al.* 2000). In addition, BMP-2 appears to have anabolic effect on human bone synthesis by inhibiting MMP-1 (Takiguchi *et al.* 1998). In view of that, the same may also be true for dentin matrix, since BMP-2 decreased the MMP-1 mRNA in odontoblasts. However, when TGF-β1 and BMP-2 were combined, the opposite effect on MMP-1 mRNA expression was observed, and a trend of up-regulation of MMP-1 transcription could be detected in odontoblasts (Fig. 5). Whether this observation is because in combination

these growth factors have the ability to oppose each other, as has previously been suggested (Centrella *et al.* 1995), remains to be further analyzed.

Pulp tissue is a soft connective tissue containing various cell types. Even though dentin, odontoblasts and pulp tissue are often referred to as a functional dentin-pulp complex (Torneck 1994), with respect to cellular function, the pulp tissue must be considered as a separate entity. The effect of growth factors is dependent on tissue and cell type (Duivenvoorden *et al.* 1999). This was also seen in the dentin-pulp complex, as MMP-1 and MMP-13 were significantly induced by BMP-2, and to a lesser extent, by TGF-β1 in pulp tissue, but decreased in odontoblasts (see Figs 5, 6). Previously, TGF-β1 has been shown to down-regulate cytokine-induced MMP-1 mRNA in cultured pulp fibroblasts (Tamura *et al.* 1996). The different responses in these studies may indicate differential effects of TGF-β1 on pulp tissue under healthy or inflamed conditions. Alternatively, they may reflect the differential responses of tissues and cell cultures, as was observed previously with TGF-β1 effects on type I collagen (I). The results further underline the importance of culture conditions on pulp tissue studies.

6.2.2 Stromelysins (IV, Figure 7)

MMP-10 was copiously expressed by mature human odontoblasts, and it is also expressed in other mineralized matrix producing cells including osteoblasts and chondrocytes (Bord *et al.* 1999). While MMP-3 protein has been detected in human bone matrix and in rat predentin (Bord *et al.* 1999, Hall *et al.* 1999), MMP-3 mRNA was not detected in native human odontoblasts or pulp tissue, not even by a highly sensitive nested-PCR method (IV). However, the expression was detected in cultured odontoblasts and pulp tissue, demonstrating that MMP-3 gene transcription can be activated in dentin-pulp complex cells. However, another stromelysin, MMP-10, was abundantly expressed in the odontoblasts. Since MMP-10 and MMP-3 have marked structural and functional similarities, and they share a large number of substrates, it is likely that in healthy human teeth, MMP-10 is the stromelysin responsible for the regulation of dentin mineralization, as suggested for MMP-3 in rat incisors. This would explain the apparent differences in MMP-3 expression between human (IV) and rat (Hall *et al.* 1999) odontoblasts.

A slight trend could be seen that MMP-10 was induced by TGF-β1 in odontoblasts, even though the finding was not statistically significant (Fig. 7). There are only a few studies on the regulation of MMP-10 by TGF-β, and at least in keratinocytes TGF-β1 induces the expression of MMP-10 (Madlener *et al.* 1996, Rechardt *et al.* 2000). In pulp tissue, BMP-2 significantly induces the expression of MMP-10, similar to its induction of MMP-1, MMP-2 and MMP-13.

6.2.3 Other MMPs (IV, Figure 8)

The dentin-pulp complex cells produce several other MMPs, of which MMP-20 is predominantly expressed in mature human odontoblasts compared to pulp tissue (IV, Fig. 8), as has been indicated (Llano *et al.* 1997, Sulkala *et al.* 2002). The present work indicates that TGF-β and BMP-2 do not regulate MMP-20 mRNA expression in mature odontoblasts and pulp tissue (IV). The expression of MMP-20 is almost exclusively limited to dentin-pulp complex cells. There is only one study showing that MMP-20 is expressed by tongue carcinoma cells *in vitro*, and comparable to our finding, TGF-β1 does not affect its expression in these cells (Väänänen *et al.* 2001).

MMP-12 was not detected in either odontoblasts or pulp tissue, regardless of the presence or absence of growth factors. Kerkelä and colleagues (2001) also have not detected MMP-12 in adult normal cartilage. This is in line with the current understanding that MMP-12 expression almost exclusively restricted to macrophages (Belaaouaj *et al.* 1995).

The expression of MMP-19 in the dentin-pulp complex cells, especially in odontoblasts, is interesting since it has previously been shown to be mainly expressed in several internal organs (Pendas *et al.* 1997b). In addition to MMP-19, this is the first example showing the expression and regulation of MMP-27 by odontoblasts and pulp tissue. However, the function and regulation of MMP-27 is still unknown. Therefore, its role in the dentin-pulp complex cannot be speculated at this point.

6.2.4 Gelatin-binding MMPs (IV, Figure 9)

Previously, both MMP-2 and MMP-9 have been shown to be secreted by mature human odontoblasts (Tjäderhane *et al.* 1998b), and MMP-2 is also expressed by human osteoblasts (Rifas *et al.* 1989), and MMP-9 by osteoclasts, osteoblasts and chondrocytes (Okada *et al.* 1995, Festuccia *et al.* 2000, Thompson *et al.* 2001). This thesis further confirmed these findings, and showed that mRNAs of both MMP-2 and MMP-9 were expressed by odontoblasts and pulp tissue (IV, Fig. 9). Both latent and active protein forms of MMP-2 and MMP-9 were observed in conditioned odontoblast culture media, but strikingly, both gelatin-binding MMPs were detected only as latent forms in conditioned pulp tissue culture media (IV). Whether this is due to a lack of appropriate zymogen activators is unknown, but there is recent indication that at least MMP-9 is secreted from human pulp tissue and mouse osteoblasts as a latent enzyme (Mizutani *et al.* 2001, Chang *et al.* 2001). Furthermore that latent MMP-9 exhibits proteolytic acitivity, capable of degrading gelatin (Bannikov *et al.* 2002), providing a new insight into proMMPs functons. It remains to be studied whether other MMPs also possess functional activity in their proforms, and what is the possible functional significance of this activity in tissues in general, and in the dentin-pulp complex.

TGF-β1 and BMP-2 differentially regulate gelatin-binding MMPs. As previously detected in odontoblasts, osteoblasts and chondrocytes, MMP-9 expression is up-regulated by TGF-β1 (Tjäderhane *et al.* 1998b, Festuccia *et al.* 2000, Thompson *et al.*

2001). The present work also shows that MMP-9 mRNA is significantly induced by TGF-β1 in odontoblasts. In addition, the combination of growth factors significantly induced MMP-9 mRNA, and also MMP-2 mRNA expression in the odontoblasts. TGF-β1 alone did not affect MMP-2 mRNA expression, in line with previous findings (Tjäderhane *et al.* 1998b). There are indications that MMP-2 may not be constitutively expressed by mineralized tissue forming cells, since in osteoblasts cytokines and IGF-1 induce MMP-2 (Damiens *et al.* 2000). In pulp tissue, growth factors did not regulate MMP-9 mRNA expression, but significantly induced MMP-2 (IV, Fig. 9). These findings have also been observed with cultured pulp fibroblasts (Chang *et al.* 2001).

6.2.5 Furin-activated MMPs (IV)

The expression of MMP-11 in the dentin-pulp complex is interesting, since there are no classical ECM substrates for human MMP-11. However, the expression of MMP-11 mRNA in odontoblasts and pulp tissue seemed to be low, since its expression in the tissue culture conditions was below detection level.

6.2.6 Minimal-domain MMPs (IV, Figure 10)

The present study shows for the first time that minimal-domain MMPs, MMP-7 and MMP-26, are expressed by odontoblasts or pulp tissue. In addition, a trend that MMP-7 mRNA could be slightly induced by either TGF-β1 or BMP-2, and MMP-26 mRNA by both growth factors in combination was observed. MMP-7 has also been shown to be induced by TGF-β1 in glioma cells, but inhibited in epithelial cells (Nakano *et al.* 1993, Bruner *et al.* 1995), indicating that MMP-7 regulation is tissue specific. Information on the regulation of MMP-26 in cells other than odontoblasts and pulp tissue is not currently available.

6.2.7 Type I transmembrane MMPs (III, IV, Figure 11)

MMP-14 is highly expressed in bone and cartilage (Buttner *et al.* 1997, Sato *et al.* 1997). Mature human odontoblasts and pulp tissue also expressed MMP-14 mRNA and synthesized latent MMP-14 protein (III). The active form of MMP-14 was also detected both in odontoblasts and pulp tissue, indicating that the activation of MMP-14 at the cell membrane detected in other tissues (Sato *et al.* 1994) may also occur in the dentin-pulp complex cells. In addition to MMP-14, the other type I transmembrane MMPs, MMP-15 and MMP-16, were expressed by both odontoblasts and pulp tissue (IV, Fig. 11).

This work also shows for the first time the effect of TGF-β-superfamily members on MT-MMPs gene regulation in mineralized tissue, and reveals that TGF-β1 and BMP-2

down-regulated the mRNA expression of MMP-14 in both odontoblasts and pulp tissue. In the case with other odontoblast-derived MT-MMPs, the opposite effect was observed, and the combination of the TGF-β1 and BMP-2 stimulated rather than decreased mRNA expression (Fig. 11). This differential effect of the growth factors on MT-MMP subgroup members may reflect differential gene promoter structure, and hence variable responses to stimuli, and possibly different roles in the ECM milieu. However, detailed information of the structure of each gene is not available, and their precise role is still unclear.

6.2.8 GPI-linked MMPs (IV, Figure 12)

The present thesis shows that in the dentin-pulp complex MMP-17 is exclusively expressed by pulp tissue, whereas MMP-25 is expressed abundantly by odontoblasts compared with pulp tissue (IV, Fig. 12). This study also shows for the first time the effects of TGF-β1 and BMP-2 on both MMP-17 and MMP-25 gene regulation, indicating that TGF-β1 and BMP-2 are likely to induce MMP-17 mRNA expression in pulp tissue. This is consistent with findings in other pulp tissue derived MMPs, such as MMP-1, MMP-2 and MMP-13, which are also up-regulated rather than down-regulated by growth factors used in these experiments (Figs 5, 6, 9). However, MMP-25 was not distinctly affected by these growth factors (Fig. 12).

6.2.9 Type II transmembrane MMPs (IV, Figure 13)

MMP-23 mRNA was expressed more abundantly in odontoblasts than in pulp tissue (IV). The expression was induced by the combination of TGF-β1 and BMP-2 in odontoblasts (Fig. 13). There are no previous studies of the expression of MMP-23 in the dentin-pulp complex cells, and further studies are needed to determine the functional significance of this enzyme in the dentin-pulp complex.

6.3 Expression and regulation of TIMPs in human dentin-pulp complex (IV, Figure 14)

One of the primary roles of TIMPs is to specifically regulate the activation and activity of MMPs. Since numerous MMPs are produced in dentin-pulp complex cells, it is predictable that their natural inhibitors would also be expressed by these cells to control the unrestrained catalytic activities of the tooth-derived MMPs. Indeed, the present thesis shows for the first time that in addition to previously detected TIMP-1 (Ishiguro *et al.* 1994), TIMP-2, TIMP-3 and TIMP-4 are also expressed by mature human odontoblasts and pulp tissue (IV, Fig. 14). Other studies have indicated that TIMP-1 and TIMP-3 expression are induced by TGF-β1 and BMP-2 in calcified tissues (Overall *et al.* 1995,

Su *et al.* 1996, Varghese & Canalis 1997, Wang *et al.* 2002). In agreement with these studies, TGF-β1 in combination with BMP-2 induced the expression of TIMP-1 and TIMP-3 mRNA in odontoblasts (Fig. 14). Comparable effects were also shown for TIMP-3 in pulp tissue. TIMP-2 gene transcription was not regulated by the growth factors studied in dentin-pulp complex, as has been observed by Ihn and colleagues (2002) with dermal fibroblasts. However, TIMP-2 may still be transcriptionally regulated, since there are evidences that cytokines induce TIMP-2 expression, at least in dermal fibroblasts (Ihn *et al.* 2002). Strikingly, we found that contrary to other TIMPs, TIMP-4 mRNA expression was significantly inhibited by TGF-β1 and BMP-2 in pulp tissue (Fig. 14), suggesting that TIMP-4 may have a different role during dental injury than the other three TIMPs.

6.4 ProMMP-20 activation (III)

The process of activation of latent MMPs serves a critical regulatory step for controlling the ability of MMPs to degrade the ECM. The expression of MMP-20 is known to be practically restricted to dental tissues. A recent study demonstrated its precence in dentin matrix and dentinal fluid, and indicated that MMP-20 could be involved dentin-pulp complex defensive reactions (Sulkala *et al.* 2002). However, the present work shows that the MMP-20 gene is not regulated by TFG-β -superfamily members. The lack of growth factor regulation of MMP-20 expression is further supported by the *in vivo* finding that demonstrates presence of MMP-20 protein equally in healthy and carious teeth odontoblasts (Sulkala *et al.* 2002). The latent MMP-20 was converted into the active form by another active MMP, MMP-14 (MT1-MMP), as has also been shown for proMMP-2 activation (Sato *et al.* 1994, Llano *et al.* 1999). This suggests that odontoblast-derived MMP-20, which is constitutively secreted in a latent zymogen form by odontoblasts, requires the activation mediated by MMP-14 in the cell surface in order to function in dentin matrix formation and mineralization.

6.5 Possible roles of MMPs and TIMPs in human dentin-pulp complex in health and disease

This thesis shows that mature human odontoblasts and pulp tissue are capable of expressing various MMPs and TIMPs, in addition to those observed earlier: MMP-2, MMP-9 and MMP-20. Furthermore, TGF-β1 and BMP-2 differentially regulate the expression of these proteins. It is still unknown why so many members of different subclasses of metalloproteinases are synthesized in dentin-pulp complex cells, and what their functions might be in human dentin-pulp complex. There are only a few studies demonstrating the role or function of an individual MMP in the dentin-pulp complex *in vivo* in physiological or pathological conditions (Hall *et al.* 1999, Wahlgren *et al.* 2002).

MMPs may have dual-roles; they can act in both healthy and disease conditions. Thus, the possible roles of MMPs in mature odontoblasts could be classified in the following subcategories: 1) Functions of MMPs in intact and healthy teeth in processes of physiological secondary dentin formation and mineralization, 2) MMPs participating in matrix degradation during dental injury, 3) Roles of MMPs in tertiary dentinogenesis, and 4) Roles of MMPs in pulpal inflammation.

6.5.1 MMPs and dental matrix synthesis and mineralization

MMPs are suggested to play a role in the initiation of cartilage mineralization (D'Angelo *et al.* 2001). During dentin matrix mineralization, MMPs may take part in collagen matrix organization, before mineral deposition occurs. During the organization of collagen fibrils other proteins such as proteoglycans are involved (Jones & Boude 1984, Boskey 1989), and if they need to be cleaved prior to mineralization, it is most probably done by MMPs. There is evidence that hydroxyapatite crystals inactivate and induce autocatalytic degradation of both MMP-1 and MMP-3 *in vitro* (Kremer *et al.* 1998). Therefore, it is possible that the MMP activity during predentin matrix degradation is at least partially regulated by the formation of hydroxyapatite crystals at the mineralization front.

So far, there is only one histological analysis showing that MMP-3 is located in the predentin, and thus, it is suggested to participate to the regulation of dentin mineralization by regulating the presence and activity of different proteoglycans in the different parts of predentin (Hall *et al.* 1999). However, this work is done with continuously growing rat incisors, and since we did not detect MMP-3 in mature human odontoblasts, it is possible that in humans another member of stromelysin family, MMP-10 (stromelysin-2), is responsible for the function suggested for MMP-3 in rat incisors, since relatively high expression levels were detected for this MMP in the odontoblasts (Table 2). Therefore, it is possible that significant differences occur in the MMP expression profiles in dentin-pulp complex between species.

6.5.2 MMPs and dental injury

If a tooth is affected by an injury and dentin demineralization occurs, which leads to a perturbation of tooth homeostasis, and possibly to uncontrolled activities of MMPs, it is highly possible that TGF-β1 may participate in the regulation of different dentin-pulp complex defensive reactions. TGF-β1 may, for example, protect the pulp tissue from degradation in a similar way that it protects cartilage collagen from destruction, by reducing collagenases (Hui *et al.* 2000). Since glycosaminoglycans and decorin, which both exist in dentin, are able to bind growth factors (Yoshiba *et al.* 1996, Imai *et al.* 1997), and latent TGF-β is stored in the ECM (Dallas *et al.* 1995), it is possible that MMPs bound to dentin matrix serve as a reservoir of enzymes capable of releasing matrix bound latent TGF-β1 and activating it (Allan *et al.* 1991, 1995, Imai *et al.* 1997, Martin-

De Las Heras *et al.* 2000, Yu & Stamenkovic 2000). Thus MMPs may be involved in the TGF-β mediated cellular responses occuring during dental injury. However, as a response to injury, TGF-β may not stimulate type I collagen synthesis (I), but may indirectly affect dentin matrix synthesis by regulating, alone or synergistically with BMP-2 and possibly other dentin-bound growth factors, the expression of MMPs. Indeed, differential effects on several MMPs for both growth factors were detected in these (II, III, III) and in previous studies (Tjäderhane *et al.*1998b). During prolonged dental injury, it is highly probable that TGF-β may induce BMP-2 expression, as has been detected with pulpal cells (Calland *et al.* 1997). Together they may have a positive role in promotion of pulpal healing, and this effect may be at least partially mediated by the regulation of MMP expression and synthesis.

If the dental injury is severe and leads to odontoblast cell death, TGF-β may induce new odontoblast-like cell differentiation from pulpal stem cells and their migration to the pulp chamber edge, where they are responsible for dental collagen matrix synthesis and mineralization (D'Souza *et al.* 1995, Melin *et al.* 2000). In the present study, TGF-β1 and BMP-2 seem to only induce the expressions of pulpal MMPs of MMP-1, -2, -13 and -17, of which MMP-13 and MMP-17 are expressed at much higher levels by pulp tissue compared to odontoblasts. Since the expression level of MMP-8 was quite low in the pulp tissue compared to MMP-13 (IV), it is possible that MMP-13 is the main collagenase in the pulp tissue together with MMP-1. This is supported by the recent study utilizing cDNA microarray, in which MMP-13 exprssion was extremely high in the pulp tissue compared to all the other MMPs studied (Sulkala *et al.* submitted for publication). Although the steady state expression of MMP-1 is low in the healthy tooth (IV), growth factors induced the expression of MMP-1. Since pulp tissue inflammation is an essential defensive reaction in the protection of dentin-pulp complex, and since growth factors mainly induced the expression of MMPs, it is possible that MMPs may have a role during pulpal inflammation, by mediating the breakdown of the pulpal connective tissue enabling migration of the odontoblast-like cells, as has been suggested for MMP-2 in the induction of epithelial cell migration (Giannelli *et al.* 1997).

6.5.3 TIMPs in physiological and pathological conditions of teeth

TIMPs have widespread biological functions in addition to MMP-inhibition, *e.g.* they may stimulate cell growth (Hayakawa *et al.* 1992, Nemeth *et al.* 1996). Since the present study shows that TIMP-2 is not regulated by TGF-β and BMP-2 (see Fig. 14, Table 2), it is possible that TIMP-2 is constitutively expressed in the dentin-pulp complex, and one of its physiological functions may be activation of MMPs, as has been indicated with MMP-2 (Wang *et al.* 2000). Whether TIMP-2 is also included in the MT1-MMP mediated proMMP-20 activation remains to be studied. TIMP-1 is able to bind collagen fibrils bound active MMP-1 (Allan *et al.* 1991), and since TGF-β1 and BMP-2 induce the expression of TIMP-1 in human odontoblasts, it is highly possible that TIMP-1 may indirectly inhibit collagen matrix destruction by inhibiting active MMP-1. Furthermore, since type III collagen is the main substrate of MMP-1, and type III collagen is a component of a reparative dentin, it is possible that as a response to exernal irritation,

type III collagen in the predentin is no longer degraded by MMP-1, allowing dentin mineralization to proceed to reparative dentin.

Of the TIMPs, TIMP-3 has the ability to bind to the ECM, especially to matrix components containing glycosaminoglycan chains, such as heparin, and sulphated components (Langton *et al.* 1998, Yu *et al.* 2000). TIMP-3 may serve as a reservoir of inhibitors in the dental matrix, and during dental injury it may be released from the matrix to control activities of MMPs. It is possible that TGF-β is released from the matrix during dental injury, and since we show that TGF-β1 in the pulp tissue alone, and together with BMP-2 in odontoblasts induces TIMP-3, it is possible that TIMP-3 released from the matrix inhibits the activities of MMPs during dental injury. In addition, TIMP-3 may also induce cell apoptosis (Bond *et al.* 2000). The possible role of TIMP-3 in apoptosis of odontoblasts as a response to severe caries would be an interesting hypothesis to study.

Contrary to other pulpal TIMPs, the present study shows that TIMP-4 expression is inhibited by TGF-β1 and BMP-2. Since TIMP-4 is a general inhibitor of many MMPs (Stratman *et al.* 2001), it is possible that during dental injury inactivation of TIMP-4 permits the possible MMP mediated matrix degradation during pulpal cell migration, indicating that TIMP-4 may have an indirect healing effect on dentin matrix as a response to injury. For example, TIMP-4 regulates the activity of MMP-2 either directly or through inhibition of MT1-MMP (Bigg *et al.* 2001, Hernandez-Barrantes *et al.* 2001). Since the growth factor down-regulation of TIMP-4 seemed to be accompanied by the induced expression of MMP-2 (Table 2), TIMP-4 may have a role in the increased activity of MMP-2 in pulp tissue (Table 2).

6.5.4 Other possible roles for MMPs and TIMPs in the dentin-pulp complex

Even though the best-known effect of MMPs and TIMPs is the degradation of proteins either in morphogenesis and growth of tissue or associated with tissue catabolic activities, their role is not limited to protein degradation as such (reviewed in Sternlicht & Werb 2001, Lopez-Otin & Overall 2002). It has been increasingly appreciated that by highly specifically recognizing and hydrolysing their substrates, proteinases are important regulators of diverse biochemical reactions both within the cell and in the ECM. Proteolysis is more than simple degradation; it is an important mechanism to regulate activities of proteins, for example by controlling their shedding from the cell, and their localization. Furthermore, proteinases may activate or inactivate other proteinases, enzymes, growth factors and cytokines, and hence they have central role in diverse biological events such as in cell proliferation, differentiation and migration, and tissue remodelling and apoptosis (reviewed in Lopez-Otin & Overall 2002). There is also increasing evidence that TIMPs also have roles other than regulation of MMP-activity. They have, for example, growth factor-like activity and can inhibit angiogenesis (reviewed in Gomez *et al.* 1997). Therefore, the roles of individual MMPs and TIMPs, either alone or acting in different combinations, may prove to be much more diverse than what we know today in many tissues, including the dentin-pulp complex.

7 Future prospects

The present thesis, showing the multiple expressions of MMPs and TIMPs in mature human odontoblasts and pulp tissue, and that they are differentially regulated by TGF-β1 and BMP-2, gives further important information on both mature human odontoblasts and pulp tissue secretory activities. In addition, this thesis indicates that the growth factors may not regulate the synthesis of collagens in mature human odontoblasts, but that they may indirectly regulate dentin and pulp tissue matrix synthesis and breakdown through a complex regulatory cascade of steady state levels of MMPs and TIMPs. However, the precise roles of these proteins in dental tissue still remain unclear. Since proteinases do not function in isolation, but rather in conjunction with other proteinases and their substrates and cleavage products, as well as inhibitors, it will be important to focus on the this whole system to provide more information on the biological events in cells and their tissue environment (Lopez-Otin & Overall 2002). Thus, more detailed analysis of the function and importance of each MMP and TIMP in dentin matrix synthesis prior to mineralization are needed. In this task, current and future degradomic techniques such as protease-specific protein chips, protease activity chips and substrate chips, and many others such as DNA microarray chips, offer valuable tools (Lopez-Otin & Overall 2002). The first step in the future studies should be to confirm the location and activity of different MMPs and TIMPs in healthy and diseased dentin-pulp complex tissue. Also, further analysis of other growth factors and cytokines are needed to widen knowledge of regulatory mechanisms. And finally, *in vivo* studies aiming to selectively regulate MMP-TIMP –expression and activity in the dentin-pulp complex may open a pathway for improved means to master pulpal diseases in clinical work.

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